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A novel extracellular cold-active esterase of *Pseudomonas* sp. TB11 from glacier No.1: Differential induction, purification and characterisation

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ABSTRACT

The production, purification, characterization and application of a novel cold active esterase by *Pseudomonas* sp. TB11 are described herein. A new finding regarding the production of extracellular esterase activity depending upon single cream as an inducer used for growth was investigated in this study. The crude esterase was subjected to a three-step enzyme purification, which resulted in a 15.21-fold purification and the specific activity of the final purified esterase increased to 1526.2 U/mg protein and purified EstTB11 had a molecular mass of 65 kDa. The N-terminal sequence of ten amino acids were: GVYDYKNLTT. Peptide mass finger printing revealed that some peptides showed homologues sequences (29%) to polyurethanase of *Pseudomonas* sp. FH4. Furthermore, the enzyme displayed the optimum pH of 8.5 and optimum temperature of 25 °C and significantly high stability at 15–35 °C for 72 h. The enzyme was incubated with different metal ions at concentrations of 5 and 10 mM, the activity of esterase was increased in the presence of K^+, Na^+ and Mg^{2+} and decreased with Ca^{2+}, Al^{3+}, Mn^{2+}, and Fe^{3+}. Experiments indicated that EstTB11 could hydrolyze milk fat to produce short and medium-chain fatty acid and this result layed the foundation for the application in increased aroma of milk products.

1. Introduction

Glacier No. 1 has an elevation range of 3730–4486 m a.s.l. and is located at the headwaters of the Urumqi River in Tianshan, China (43°05′N, 86°49′E) [1]. It is a perennially dry and cold zone with an annual average temperature of approximately −5 °C and serves as a vast bioresource repository of cold-adapted microorganisms. Moreover, recent researchs particularly on cold-active enzymes, herald the rapid growth in this burgeoning field [2]. Thus, it is crucial to develop the investigation on microorganism resources of glacier No. 1, which has received a tremendous amount of attention in the research of extremophiles [3]. Enzymes produced from psychrophilic and psychrotolerant microorganisms in permanently cold habitats could be better adapt to the low temperatures. When compared to the mesophilic counterparts, cold-adapted enzymes display a higher catalytic efficiency over a temperature range of roughly 0–40 °C.

Esterases/lipases have been recognised as useful biocatalysts due to the success of certain applications, including those in the oleochemical and detergent industries, the production of biodegradable polymers, food flavouring, waste treatment, oil biodegradation, and biodiesel production [4,5]. The high catalytic activity at low temperatures can also be favorable in their application [6]. Esterases differ from lipases mainly based on their substrate specificity and interfacial activation. In fact, lipases prefer water-insoluble substrates, typically triglycerides composed of

Abbreviations: EstTB11, a cold-adapted esterase from psychrotolerant *Pseudomonas* sp. TB11; *p*NPA, *p*-nitrophenyl acetate; *p*NPB, *p*-nitrophenyl butanoate; *p*, NPC*p*-nitrophenyl caprylate; *p*NPD, *p*-rophenyl decanoate; *p*NPL, *p*-nitrophenyl laurate; *p*NPP, *p*-nitrophenyl palmitate; *p*, NPS*p*-nitrophenyl tearate; ARTP, atmospheric and room temperature plasma; PVA, polyvinyl alcohol; *p*NP, *p*-nitrophenol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; PVDF, polyvinylidene difluoride; SPME, solid-phase microextraction; GC/MS, gas chromatography/mass spectrometer.

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long-chain fatty acids, whereas esterases preferentially hydrolyze short or medium-chain fatty acids.

Although several studies have been performed deep in the sea [7], the Antarctic [8,9], the western Himalaya mountains [10], little is known with respect to cold-adapted esterases in glacier resources, especially in the Tianshan Mountains. An extracellular esterase, EstK, was previously purified from a cold-adapted *Pseudomonas mandelii*. The enzyme EstK was active at low temperatures and showed substrate preference for short-chain fatty acids, especially *p*-nitrophenyl acetate (*p*NPA) [11]. A psychrotolerant bacterium, named strain T1-39 was obtained by carrying out extensive screening for esterases producer from the low temperature microorganisms of tundra soils and glacier melt warters in glacier No. 1, which was identified as a *Pseudomonas* sp. (accession number: KP780205). Strain TB11 was a mutant of *Pseudomonas* sp. T1-39 by atmospheric and room temperature plasma (ARTP) mutation. However, an esterase, EstT1-39, produced from *Pseudomonas* sp. T1-39 is prefer to hydrolyse esters of glycerol with short-chain fatty acids, especially *p*-nitrophenyl butanoate (*p*NPB). This kind of esterase lay a foundation for further application by hydrolyzing milk products to increase its aroma [12]. Previous work also confirmed the finding that crude EstTB11, produced from strain TB11, was stable at room temperature and highly active at low temperatures, and its characteristics made it potentially important for industrial applications.

However, at present, this poses a problem because the properties of each esterase may be different, they may exhibit different activity, stability or selectivity, and they may be affected in different ways by the experimental conditions. Moreover, the results may change between different batches of the enzyme. Therefore, pure esterase preparations are required to provide complete control and understanding of the processes. Hence, purification and characterisation are important for the production of highly active and stable esterases. Against this background, this study was focused on the production, purification,characterisation and application of a novel EstTB11 from *Pseudomonas* sp. TB11.

2. Material and methods

2.1. Material

Peptone, yeast extract, glucose and agar were purchased from Thermo Fisher Oxoid. Rape seed oil, soybean oil, olive oil, single cream, sunflower oil samples were obtained from local market. Triton X-100, gum-acacia powder, polyvinyl alcohol and triolein were obtained from Biosharp. Sodium dodecyl sulphate (SDS), *p*-nitrophenyl acetate (*p*NPA), *p*-nitrophenyl butanoate (*p*NPB), *p*-nitrophenyl caprylate (*p*NPC), *p*-nitrophenyl decanoate (*p*NPD), *p*-nitrophenyl laurate (*p*NPL), *p*-nitrophenyl palmitate (*p*NPP) and *p*-nitrophenyl stearate (*p*NPS) were obtained from Sigma Chemical Co. (China). Palatase 20,000 L was offered with Novozyme. HiTrap Q Sephrose FF ion exchange column and Superdex 75 10/300 GL gel filtration column were obtained from GE Healthcare (Co., Ltd., Hong Kong). Mini double-side vertical electrophoresis apparatus (DYCZ-24DN, Beijing, China). SCIONSQ-456-GC gas chromatograph, Bruker, with an DB-WAX fused silica capillary column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness; Agilent Inc., USA). All other chemicals in the investigation were of analytical grade and were purchased from Sinopharm chemical reagent factory (Co., Ltd., PR China).

2.2. Bacterial strain and culture conditions

Strain TB11 used in this study was a mutant of *Pseudomonas* sp. T1-39, which was isolated from frozen soil, sourced from the bottom sediments of west branch of Tianshan glacier No. 1 in Xinjiang, China, with an elevation of 3800 m (43°7′8″N, 86°48′42″E). The strain was maintained on LB agar slopes containing peptone 1%, NaCl 1%, yeast extract 0.5% and agar 2.0%. The duplicate screening medium was used as production medium which contained peptone 1%, K_2HPO_4 0.2%, $MgSO_4{\cdot}7H_2O$ 0.05%, $(NH_4)_2SO_4$ 0.1%, emulsion of olive oil 20 mL/L. The initial pH of the medium was adjusted to 9.0 with 0.1 M NaOH or HCl prior to sterilization.

2.3. Inoculum preparation and esterase production

Cells of strain TB11 were inoculated in 5 ml LB liquid medium and allowed to grow on a rotary shaker with shaking at 200 rev./min and at 15 °C for 18 h. For EstTB11 production, conical flasks (250 ml) containing 25 ml fermentation medium were inoculated with 2% inoculum and incubated on a rotary shaker at 200 rev./min at 15 °C for 32 h. The samples were removed after certain time intervals to determine esterase activity and soluble protein. The culture were collected by centrifugation at 9727 × *g* at 4 °C for 20 min and the cell free supernatant was used as the crude esterase for determination of enzyme activity under standard assay conditions.

2.4. Effect and consumption of different inducers on esterase production

The effect of different inducers by replacing olive oil in the production medium with rape seed oil, soybean oil, single cream, sunflower oil and triolein were used to establish the substrate selectivity of the enzyme. Compound emulsion was obtained at a ratio of 1:3 with these oils and 2% polyvinyl alcohol (PVA). The optimal inducer was determined by the relative activity, and on this basis, different additive amounts of the optimal inducer were experimented to determin the optimum amount.

2.5. Enzyme assay and protein estimation

Esterase activity in culture supernatants was assayed by measuring the absorbance of liberated *p*-nitrophenol at 410 nm using *p*-nitrophenyl butanoate (*p*-NPB) as a substrate by Margesin [13] with some modifications.

One esterase unit activity was defined as the amount of enzyme that produced 1 μmoL of *p*-nitrophenol (*p*NP) per min. 100 μL culture supernatant was added to 1.9 mL substrate buffer, which containing two parts, one part was dissolved *p*-NPB (3 mg/mL) and another part were 50 mmol/L Tris–HCl buffer (pH 8.5), 0.1% gum-acacia powder, 0.6% Triton X-100, then the two parts were mixed at a ratio of 1:9 [14]. The mixture was incubated at 30 °C for 15 min, then 1 mL of 95% ethanol was added to terminate the reaction. Inactive enzyme sample as a control group was treated with boiling water for 10 min. The absorbance of the samples was determined spectrophotometrically at 410 nm. Then the esterase activity of the culture broth supernatant (centrifuged at 9727 × *g* for 20 min) was measured.

Protein concentration was estimated by Bradford method with bovine serum albumin as a standard [15]. During the chromatographic purification steps, protein concentration was measured as a function of its absorbance at 280 nm.

2.6. Purification of esterase TB11

2.6.1. Ammonium sulphate precipitation and dialysis

The crude esterase was purified by ammonium sulphate precipitation followed by a series of column chromatography separations at 4 °C. The cell free supernatant was used for esterase purification and ground ammonium sulphate was slowly added with constant stirring up to 60% saturation. This solution was allowed

to stand overnight at 4 °C. The precipitate was collected by centrifugation (9727 × *g* for 30 min at 4 °C) and resuspended in 50 mM Tris–HCl buffer, pH 8.5. Then resuspension solution was transferred into dialysis tubing, having 8–14 kDa cut off value, and dialyzed to remove excess salts against the same buffer at 12 h intervals. After 24 h, enzyme solution was taken out and was used for next step of purification.

2.6.2. Ion exchange chromatography purification

Esterase activity of each fraction was determined. The most active components were loaded onto a HiTrap Q-Sepharose Fast-Flow column (1.6 × 2.5 cm, GE Healthcare, USA), which had been equilibrated previously with buffer A (50 mM Tris–HCl buffer, pH 8.5), at a flow rate of 5 mL/min. Elution curves were monitored at 280 nm. Unbound protein was washed out with buffer A. Retained protein was eluted using stepwise ionic strength gradients composed of buffer A and buffer B (50 mM Tris–HCl buffer, 1 M NaCl, pH 8.5): 0–0.4, 0.4–0.60 and 0.60–1.0 M NaCl. Tubes corresponding to each fraction were pooled separately, desalted and their enzyme activities and protein contents were determined. Active fractions containing high esterase activity were pooled and concentrated using a 10 kDa Amicon Ultra tube (Millipore, USA). The concentrated protein was applied to a Superdex 75 10/300 GL gel-filtration column (1.0 × 30 cm, 23.562 mL, GE Healthcare, USA). Buffer C (50 mM Tris–HCl, pH 8.5) was used for column pre-equilibration, sample loading and elution, and the flow rate was 0.8 mL/min. The active fractions were pooled and assayed for next characterisation. The esterase preparations (crude and purified fractions) were tested on SDS-PAGE gels.

2.7. Molecular mass determination by SDS-PAGE and zymography

The purity of the isolated protein and molecular weight of pure enzyme were determined by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) on a discontinuous gel containing 5% stacking gel and 12% resolving gel of pH 6.8 and 8.8, respectively. Electrophoresis was performed with 120 mV fixed voltage. After electrophoresis the gel was stained with Coomassie Brilliant Blue and the relative mobility of the purified enzyme was compared with standard proteins of known molecular weight marker (TaKaRa BIO INC). The protein marker ranging from 14.3 to 97.2 kDa was used as a standard marker for determination of molecular weight.

Zymograms allowed rapid detection of active proteins in polyacrylamide gels, hydrolase activity was also detected by a deferred assay where native polyacrylamide gels immerseed into 1% TritonX-100 for 1 h and were rinsed four times with 50 Mm Tris–HCl (pH 8.5). Incubation in substrate buffer solution containing the substrate *p*NPB at 30 °C for 1 h, proteins diffused from the gel and hydrolyzed the substrate, resulting in a clear yellow band at the position of the protein migration by Tamilarasan with some modifications [16]. In this method, the activity could only be detected when samples were not treated with β-mercaptoethanol prior to electrophoresis.

2.8. Peptide mass finger printing of esterase analysis and protein identification

The EstTB11 band in the SDS-PAGE gel was excised and submitted for in-gel digestion with trypsin. Eluted proteins were subjected to matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) to obtain their peptide mass fingerprint. Peptide sequences were matched to the sequence of the native proteins with bioinformatics tools (Matrix Science) to confirm the identity of the proteins. Peptides mass fingerprints were selected and subjected to MS/MS. The Mascot MS/MS Ion search with MS/MS data from the SwissProt database was used.

2.9. N-terminal sequence determination

To determine the N-terminal sequence the purified esterase was electroblotted with a semi-dry transfer unit on polyvinylidene difluoride (PVDF) membrane (RPN303F, GE) after electrophoresis on a 12% SDS–PAGE gel. After blotting, the PVDF membrane was stained with Coomassie Blue R-250. The esterase band was cut out and N-terminal Edman sequencing was performed on a PPSQ-30 Analysis 10 micro sequencer connected to a PPSQ-33A Protein Sequencer (SHIMADZU) and a PPSQ-30 Data Processing software was used.

2.10. Characterisation of purified esterase

2.10.1. Substrate specificity

Substrate specificity of the esterase was determined by using *p*-nitrophenyl esters of varying acyl chain lengths from C_2 to C_{18}. The following substrates were used: *p*NPA (C_2:0), *p*NPB (C_4:0), *p*NPC (C_8:0), *p*NPD (C_{10}:0), *p*NPL (C_{12}:0), *p*NPP (C_{16}:0) and *p*NPS (C_{18}:0). The substrates were dissolved in isopropanol at a final concentration of 100 μM. Reaction was carried out under standard assay conditions.

2.10.2. The effect of pH and temperature on activity and stability

The effect of pH and temperature on purified EstTB11 was investigated by using *p*-NPB as the substrate. The optimum pH of the purified enzyme was determined over a pH range of 3–10.6 at constant molarity (50 mM) in different buffers: sodium citrate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–8.0), Tris–HCl buffer (pH 8.0–9.0), glycine–sodium hydroxide buffer (pH 9.0–10.6). The assays were performed at 30 °C. Buffers were pre-incubated at 30 °C, then we added the substrate and immediately mixed before adding aliquots of the enzyme. The pH stability was studied by incubating the purified EstTB11 in selected buffers of pH range 3–10.6 for 4 h at 30 °C. The residual enzyme activity was measured by spectrophotometric assay at 30 °C, pH 8.5.

The optimum temperature of the purified EstTB11 was determined by the enzyme activity at various temperatures (0–50 °C) in 50 mM Tris–HCl buffer, pH 8.5. Buffers were pre-incubated for at least 5 min at the temperature of the assay before initiating the reaction. Thermostability was determined by incubating purified esterase in 50 mM Tris–HCl buffer, (pH 8.5) at various temperatures (15–55 °C) for 12 h and residual activity was analyzed by spectrophotometric assay at 25 °C, pH 8.5.

2.10.3. Effect of metal ions on enzyme activity

For determining the effect of metal ions on enzyme activity, enzyme assays were performed in presence of various metal ions such as Al^{3+}, K^{+}, Ca^{2+}, Fe^{3+}, Na^{+}, Cu^{2+}, Mn^{2+} and Mg^{2+} with a final concentration of 5 mM and 10 mM using *p*NPB as substrate. After 60 min incubation at 25 °C, the residual esterase activities were assayed. Residual esterase activity was calculated as a percentage of that, without effectors.

2.11. Determination of kinetic parameters

The maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) values of the enzyme were determined at temperature (25 °C) and pH (8.5) using different concentrations 0.1–10 mM of *p*NPB substrate. Enzyme kinetics reaction was started by adding 4030 U/L purified enzyme in the substrate solution. The constant values were calculated by fitting data to linear regression using Lineweaver-Burk equation plot. All the assays were performed by

standard assay conditions. Kinetic constants were calculated using Lineweaver–Burk plot (1).

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

where [S] is the substrate concentration and V is the initial reaction rate of the enzyme.

2.12. SPME-GC/MS analysis on hydrolysates of lipolytic EstTB11and Palatase 20,000L

Single cream was hydrolyzed by EsteraseTB11 and Palatase 20,000L (Novozyme) on the conditions of enzymolysis temperature at 25 °C and 50 °C, respectively, enzymolysis pH 8.0, the additive amount of enzyme 150 U/g, substrate concentration 50% and enzymolysis time 5 h. SPME-GC/MS technology was used for extracting and analyzing the volatile compounds among the enzyme products.

Five grams of enzymatic hydrolysates were placed in a 20 mL vial. The sample was stirred for 30 min at 55 °C to accelerate the equilibrium of headspace volatile compounds between the hydrolysates matrix and the headspace. Then, volatile compounds were extracted by placing a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, Supelco Inc. Bellefonte, PA) SPME fibre into the vial and exposing it to the headspace for 30 min at 55 °C. Analysis of the volatile compounds in the hydrolysates was performed using an SCIONSQ-456-GC gas chromatograph coupled with a mass detector. An DB-WAX fused silica capillary column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness) was used. The analysis was performed using helium as the carrier gas with a flow rate of 0.8 ml/min in a splitless injection mode. The oven temperature was held at 40 °C for 3 min, rose at 5 °C/min to 90 °C, then ramped to 230 °C at a rate of 10 °C/min, and maintained at 230 °C for 7 min. GC–MS was performed with an ion source temperature of 200 °C, an ionization voltage of 70 eV, and a mass range of 1–2000 amu. All the SPME/GC–MS measurements were conducted in triplicate on different samples.

The volatile compounds were identified by matching the mass spectra with those in the Wiely7.0 and NIST 2012 Library of MS spectra and the rentention index (RI) was calculated with a homologous series of *n*-alkanes (C_4–C_{28}).

2.13. Statistical analysis

Statistical analysis was performed on the datas using the analysis of variance analysis (ANOVA) with Duncan's multiple range tests. A p value of 0.05 or less was considered to be statistically significant. The analyses were conducted by SPSS 17.0 software (SPSS Inc., Illinois).

3. Results

3.1. Effect of different inducers on EstTB11 production

Six lipids were used to select the optimal inducer. The results, presented in Fig. 1A, showed that the relative activity of single cream significantly different ($p < 0.05$) which was obviously higher than those of the other five oils. Hence, the esterase production was induced by single cream, which could specifically hydrolyze milk fat to promote the formation of aroma substances. Without the addition of any inducer, its relative activity was as high as 42.8%, which showed that *Pseudomonas* sp. TB11 could secrete esterase by itself, however, its enzyme activity was lower.

The quantity of enzyme production can be effectively improved by induction under a suitable physiological state of the bacteria. As shown in Fig. 1B, the activity of esterase was the highest on single cream with consumption of 60 ml/L. Overloading with single cream is not favourable to the production of esterase because the yield of other proteins is promoted by a high concentration of lipids, which appears be the main cause of the inhibition of esterase production. As a result, the optimal addition of single cream was found to enhance esterase production in flask experiments.

3.2. Purification of extracellular EstTB11

After 32 h of culture, the cells were separated from the fermented medium by centrifugation. The crude esterase was subjected to a three-step enzyme purification process as shown in Table 1. In this study, EstTB11was purified by using ammonium sulphate precipitation (Fig. 2A) followed by HiTrap Q-Sepharose FF anion Exchange (Fig. 2B) and Superdex 75 10/300 GL gel chromatography (Fig. 2C). By this purification procedure, the enzyme was purified 15.21-fold with an overall yield of 11.78%, and the specific activity of the final purified esterase increased to 1526.2 U/mg protein (Table 1). The purified enzyme preparations were stored at −20 °C and were used for further studies to evaluate its properties.

3.3. Gel electrophoresis and zymography

The homogeneity of the eluted protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified esterase showed a single protein band of a relative molecular mass of approximately 65 kDa (Fig. 3A, lane 4). The zymogram of the purified esterase was obtained after performing Native Page in chromogenic substrate. Distinct yellow bands were observed against the white background within 30 min, as shown in Fig. 3B.

3.4. Peptide mass fingerprinting and sequencing

Gel bands were manually excised and subjected to automated in-gel chemical modification of cysteine residues with dithiothreitol and iodoacetamide followed by tryptic digestion and the digestion mixture was analysed directly by MALDI-TOF-MS. This mass spectrometric peptide mapping was compared with the theoretical maps of known proteins (Table 2); 10 peptides in the spectrum had a mass that was homologous for peptides from polyurethanase (Esterase_lipase) of *Pseudomonas* sp. FH4, resulting in a sequence coverage of 29% (178 aa for a total size of 615 aa for the polyurethanase). Seven peptides matched with polyurethanase Esterase_lipase) of *Pseudomonas fluorescens*, two peptides are homologous to the glutamine synthetase of *P. fluorescens* (fragments).

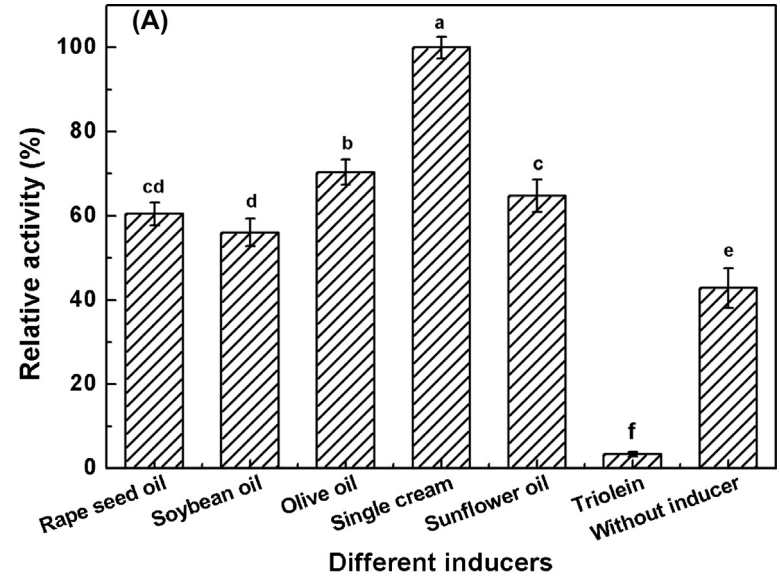
3.5. N-terminal sequence of EstTB11

N-terminal sequencing of the polyvinylidene fluoride (PVDF) transferred band from an electrophoretic gel allowed the identification of 10 amino acid residues: GVYDYKNLTT. This sequence was compared with the sequences of known esterases/lipases (Table 3). It exhibited significant similarity (80–90%) to the N-terminal sequence of other source esterases/lipases. The first 10 amino acids of EstTB11 were found to be identical to the 2–11 amino acids of a putative lipase identified from an ORF in the genome sequence of *Pseudomonas chlororaphis* O6 (genebank accession No. EIMI14990). It is likely that this ORF codes for EstTB11 as an esterase sequence that is preceded by a leader (targeting) sequence.

3.6. Characterisation of purified the enzyme EstTB11

3.6.1. Substrate specificity of purified EstTB11

The purified EstTB11 was active against a wide range of *p*-nitrophenyl esters of fatty acids with higher hydrolytic activity towards *p*-NPB and *p*-NPC, which were significantly different



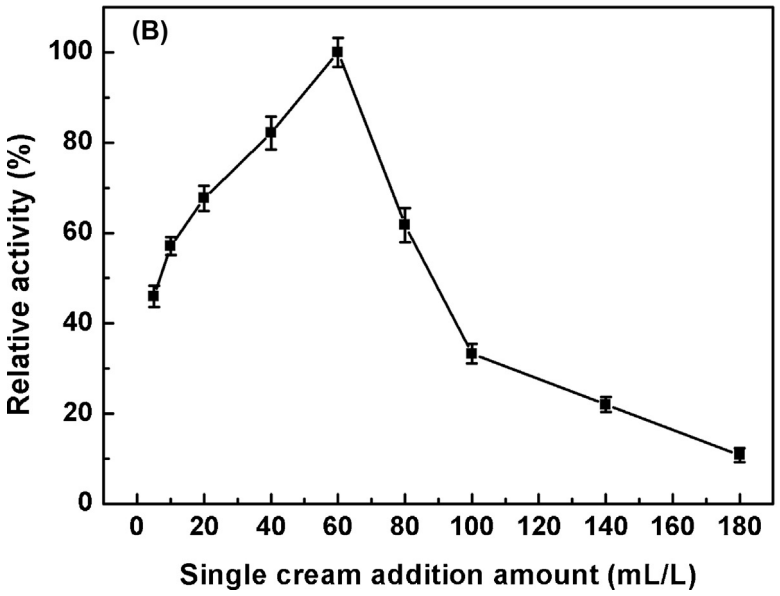


Fig. 1. (A) Effect of six inducers and without any inducer on production of extracellular esterase. (B) the optimum single cream consumption was detemined by the relative activity under the different addition amounts (5, 10, 20, 40, 60, 80, 100, 140, 180 mL/L).

Table 1
Summary of steps of purification of extracellular esterase from *Pseudomonas* sp.TB11.

Purification steps	Toalactivity (IU)	Totalprotein(mg)	Specific activity(IU/mg)	Foldpurification	Yield(%)
Culture enzyme	7644	76.2	100.31	1.00	100
Ammonium sulphate precipitation	5751.2	9.62	597.83	5.96	75.24
Q Sepharose FF	1468.48	1.80	815.82	8.13	19.21
Superdex G75	900.46	0.59	1526.2	15.21	11.78

($p < 0.05$) from the other *p*-nitrophenyl esters (Fig. 4). The results showed a preference of the enzyme for short and medium-chain fatty acids, indicating that it was more closely related to esterase. It was reported that a cold active lipase from *Pseudomonas* sp. Strain KB700A showed substrate preference towards medium acyl chain [21] and the other lipase from *Yarrowia lipolytica* had maximum activity towards *p*-NP caprylate [22].

3.6.2. Effect of pH and temperature on the esterase activity and stability

The EstTB11 characterised here were found to be functionally active in a pH range of 3.0–10.6 and showed optimum activity at pH 8.5 (Fig. 5A). It was found to be active and stable over a pH range of 7.0–10.0 at 30 °C for 240 min, which indicates residual activity up to 60% or more (Fig. 5B). The residual activities of EstTB11 was about 50% with incubation at pH 5.0 for 90 min. When the pH value

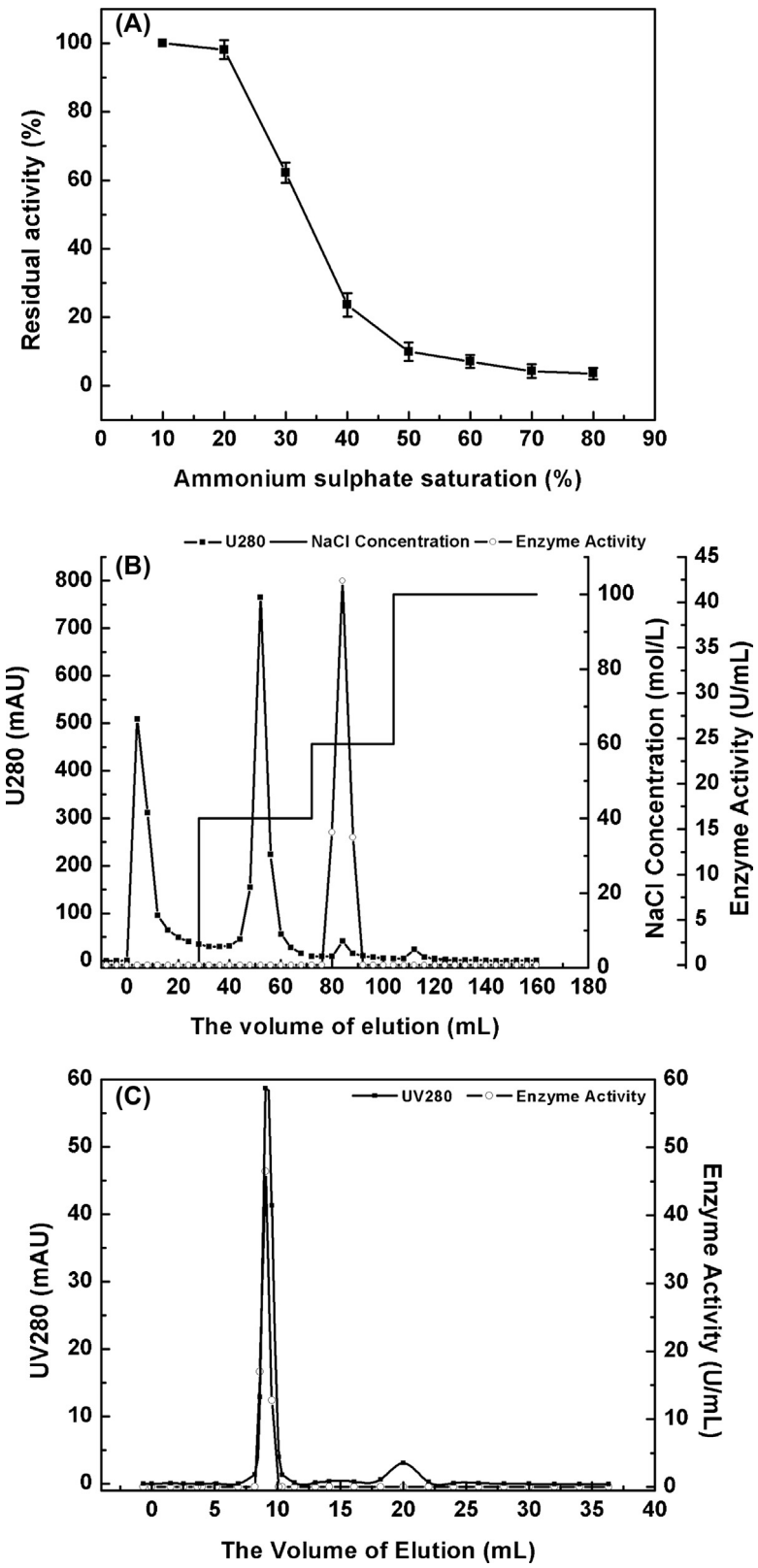


Fig. 2. Purification diagrams of EstTB11. (A) Relative of ammonium sulphate precipitation fractions; (B) Elution curve of HiTrap Q Sepharose Fast Flow chromatography; (C) Elution curve of Superdex 75 10/300 GL chromatography.

was below 5, the change in the relative enzyme activity increased until little enzyme activity remained. Thus, the pH has a obvious influence on EstTB11 activity, which is stable in both neutral and alkaline conditions.

Temperature not only influences the stability of proteins but also the maximum reaction rate of enzymes by influencing the enzyme and the substrate. The influence of temperature on esterase activity is shown in Fig. 5C and D. As can be seen in Fig. 5C, maximum activ-

Table 2
MALDI-TOF-MS and MS/MS analysis of esterase LipTB11 obtained by chemical modification of cysteine residues with dithiothreitol and iodoacetamide and tryptic digestion.

Protein	Organism	Region name	Observed	*Mr* (expt)	*Mr* (calc)	Peptide
Polyurethanase	Pseudomonas sp. FH4	Esterase_lipase	1021.4891	1020.4819	1020.4659	DGQGGISMTR
			1216.6217	1215.6144	1215.5996	ANTWVQDLNR
			1471.7900	1470.7828	1470.7678	DSTIIVANLSDPAR
			1531.6914	1530.6841	1530.6740	NTFLFDGDFGQDR
			1551.7927	1550.7854	1550.7729	VLNIGYENDPVYR
			1568.8226	1567.8153	1567.7955	DVTHSVTDQGLLAGR
			1591.8227	1590.8154	1590.8002	NVEVANDGAGTLYIR
			1641.8531	1640.8458	1640.8311	WLFGLDGNDHLIGGK
			1840.9289	1839.9216	1839.9075	GNDVLVGGAGNDLLEAGGGR
Polyurethanase	Pseudomonas fluorescens	Esterase_lipase	1591.8227	1590.8154	1590.8002	NVEVANDGAGTLYIR
			1840.9289	1839.9219	1839.9075	GNDVLVGGAGNDLLEAGGGR
			2255.1029	2254.0956	2254.0729	LVFMGVEGVDGQYNYLDHAK
Glutamine synthetase	Pseudomonas fluorescens	Gln-synt_N	1720.9245	1719.9172	1719.9018	LVPGFEAPVMLAYSAR
			2884.3655	2883.3582	2883.2995	IYSEQGSWMSDQDVEGGNHGHRPGIK
Putative calcium-binding protein	Pseudomonas sp. GM48	Esterase_lipase	1471.7900	1470.7828	1470.7678	DSTIIVANLSDPAR

Table 3
N-terminal sequence comparison of LipTB11 with homologous esterase/lipase.

Definition (accession No.)	Source/Organism	Region name	Sequence	Identities(%)	Reference
LipTB11	*Pseudomonas*	Esterase/lipase	GVYDYKNLTT (1–10)	100	This study
Polyurethanase A(EIM14990)	*Pseudomonas chlororaphis* O6	Esterase_lipase	GVYDYKNL**G**T (2–11)	90	[17]
LipA (AAF87594)	*Pseudomonas brassicacearum*	Lipase_3	G**I**YDYKNL**GTT** (2–12)	82	[18]
LipA (WP_014338392)	*Pseudomonas fluorescens*	Lipase_3	G**I**YDYKNL**G**T (2–11)	80	[19]
LipB (AAP76489)	Uncultured bacterium	Esterase_lipase	GVYDYKN**FS**T (2–11)	80	[20]

Note: Non-matching amino acid residues are underlined and highlighted in bold.

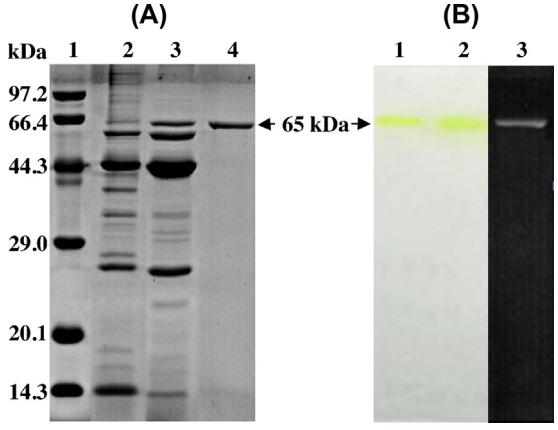


Fig. 3. SDS-PAGE of purified EstTB11 and zymogram analysis. (A) Lane 1: standard proteins; lane 2: culture supernatant; lane 3: culture supernatant precipitated with 60% ammonium sulphate and dialyzed; lane 4: purified protein after HiTrap Q Sepharose FF and Superdex 75 10/300 GL chromatography. (B) Zymogram from an Native-PAGE of purified EstTB11 analyzed for activity by *p*NPB incubation (left) and stained with silver nitrate (right). Purified EstTB11 (lanes 1–3).

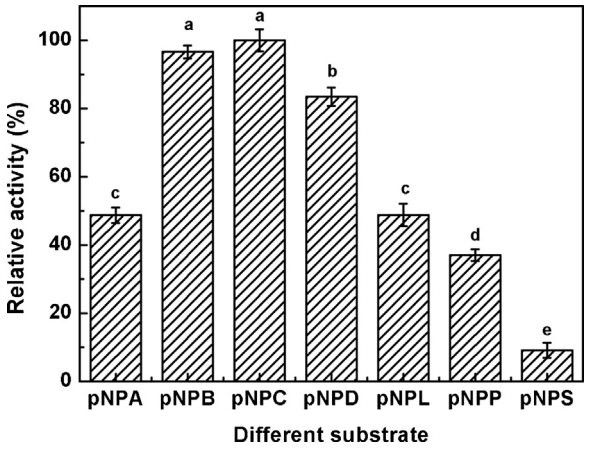


Fig. 4. Hydrolytic ability of EstTB11 towards *p*NPA, *p*NPB, *p*NPC, *p*NPD, *p*NPL, *p*NPP and *p*NPS as substrates. Set the one with the highest relative activity as 100%.

ity of EstTB11 was occurred at 25 °C while the activity of enzyme decreased gradually at temperatures below 20 °C beyond 30 °C, but it still retained more than 90% and 38% of its maximal activity at 35 °C and 0 °C, respectively.

The EstTB11 stability at 15–35 °C was stable, at around 90% at 15–25 °C and of about 80% at 35 °C for 720 min. However, the EstTB11 activity at 45 °C remained above 65% for 60 min and began to sharply decline after 90 min of incubation, with residual activity of less than 30% after 120 min. At 55 °C for 15 min, approximately 8% residual activity was observed for EstTB11, whereas it was inactivated after 240 min (Fig. 5D).

3.6.3. *Effect of metal ions on EstTB11 activity*

The presence of metal ions at 5 mM of K^+ and Na^+ promoted esterase activity for 60 min, that was about 12% to 35% greater than the control, whereas 10 mM concentration of K^+ and Na^+ enhanced the enzyme activity by 9–60% (Fig. 6). Mg^{2+} ions at a 5 mM concentration enhanced the enzyme activity by 15% more than the control; however, Mg^{2+} ions at 10 mM had no effect on esterase activity. Cu^{2+} and Ca^{2+} ions at 5 and 10 mM separately inhibited enzyme activity, but at 10 mM, Cu^{2+} ions enhanced the enzyme activity by 10% more than the control. The presence of Al^{3+} and Mn^{2+} ions at 5 mM and 10 mM concentration drastically reduced the levels of enzyme activity, which were 40–50% less than the control.

3.7. *Enzyme kinetic parameters*

The kinetic parameters K_m and V_{max} were calculated for the purified esterase from the Michaelis–Menten plot (Fig. 7). The purified esterase showed a lower K_m value (0.698 mmol^{-1}) and a higher V_{max} value (22.93 mmol/min·mg) than the esterase/lipase from the other *Pseudomonas* sp. reported in the literature [23,24]. The lower K_m value represents a higher affinity between enzymes and sub-

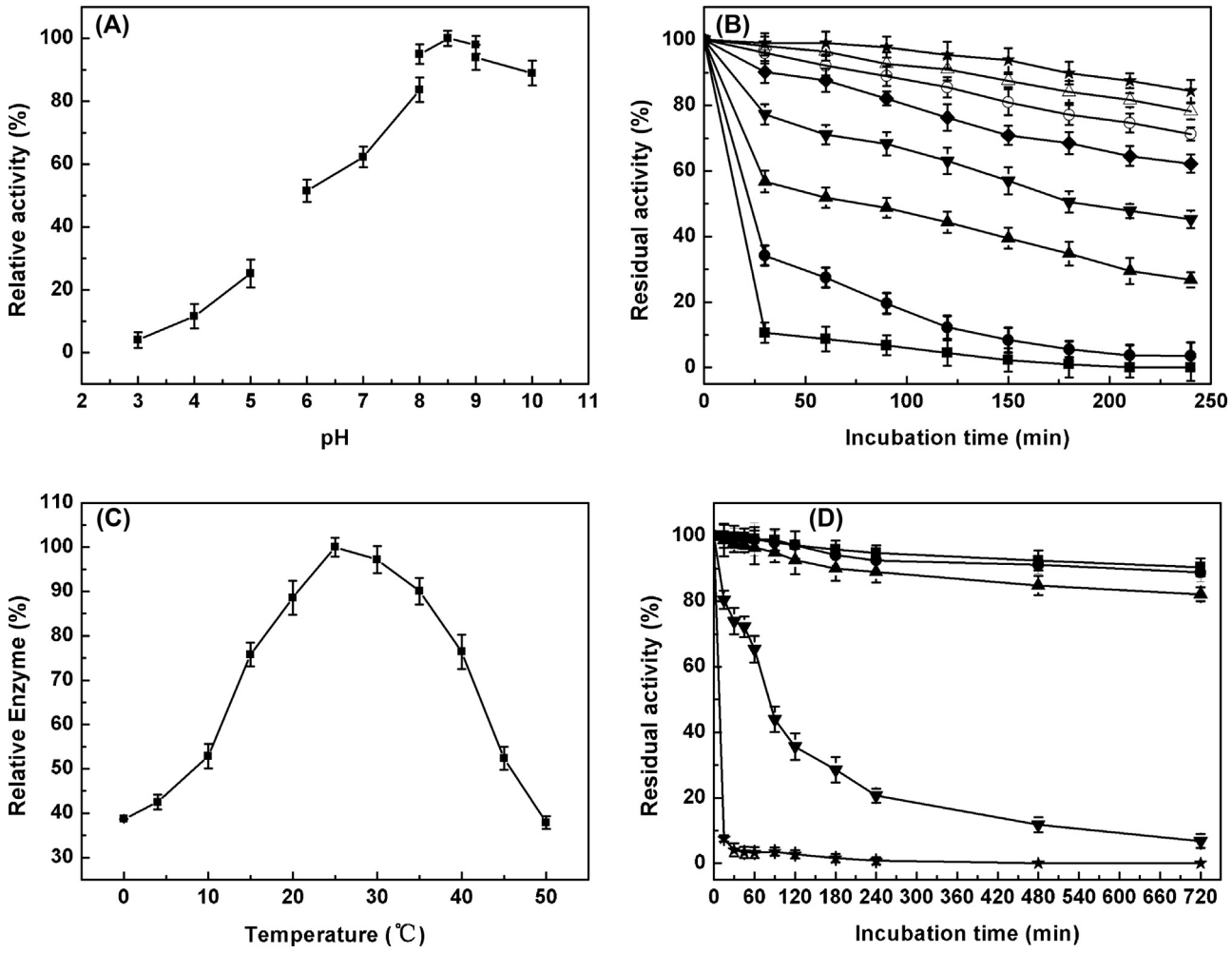


Fig. 5. (A) Effect of pH on enzyme activity by assaying in pH values(3–10.6) at 25 °C for 15 min. pH 3–5 (citric acid and sodium citrate buffer), pH 6–8 (phosphate buffer), pH 8–9 (Tris–HCl buffer), pH 9–10.6 (glycine–sodium hydroxide buffer); (B) the stability of pH was measured by adding the esterase in different pH (3–10) at 25 °C for 30, 60, 90, 120, 150, 180, 210, 240 min, respectively.
(■) pH 3, (●) pH 4, (▲) pH 5, (▼) pH 6, (○) pH 7, (△) pH 8, (⋆) pH 9, (♦) pH 10; (C) Effect of temperature on EstTB11 activities by measuring relative activity at 0–50 °C (0, 4, 10, 15, 20, 25, 30, 35, 40, 45, 50 °C) in 50 mM Tris–HCl buffer (pH 8.5) for 15 min; (D) thermostability was measured by incubating the esterase in 50 mM Tris–HCl (pH 8.5) at 15 °C (■), 25 °C (●), 35 °C (▲), 45 °C (▼) and 55 °C (⋆).

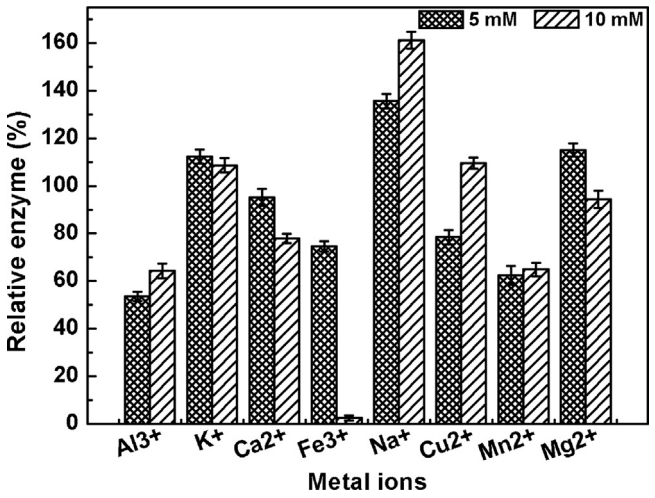


Fig. 6. The effects of different metal ions were investigated by pre-incubating the esterase with 5 mM and 10 mM final concentration of these ions at pH 8.5, temperature 25 °C for 30 and 60 min respectively. Without metal ions as the control, which was considered 100%.

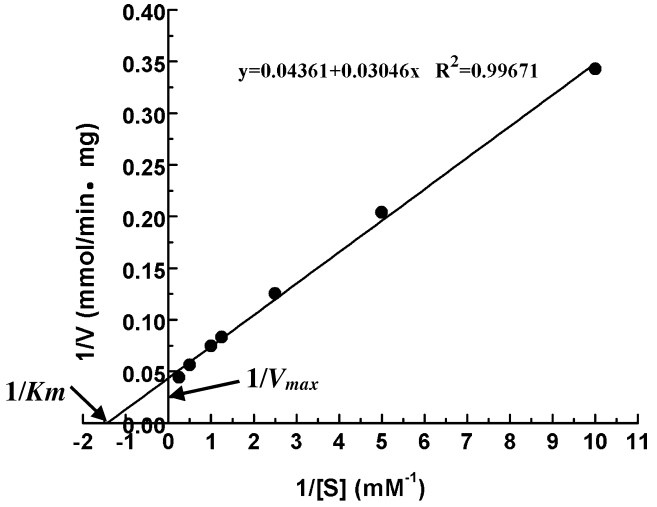


Fig. 7. Lineweaver–Burk plot of purified esterase from *Pseudomonas* sp.TB11 using *p*-nitrophenyl butanoate as a substrate.

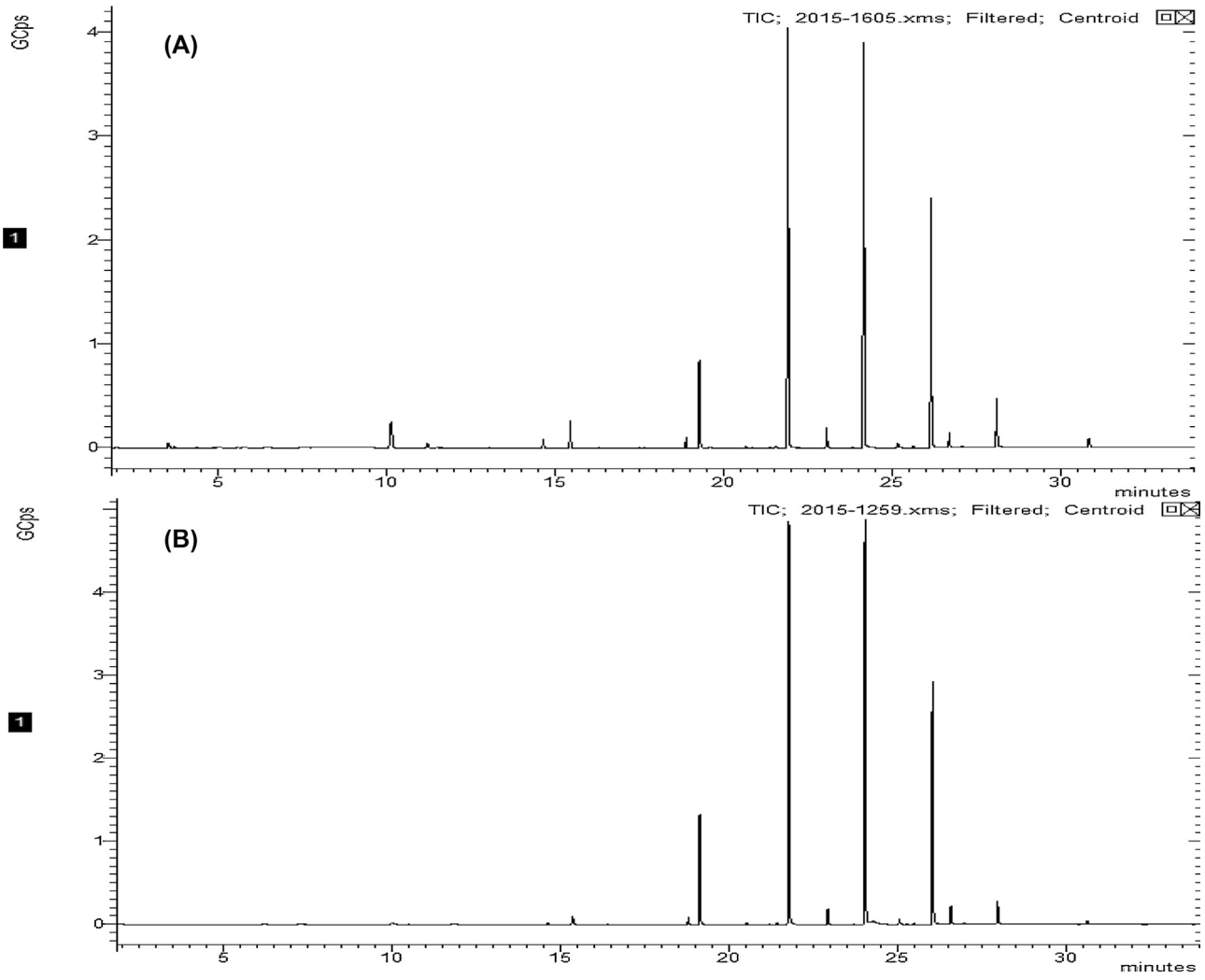


Fig. 8. Chromatograms obtained by the SPME-GC/MS analysis of the investigated compounds in two hydrolysates from EstTB11 and Palatase 20,000 L under the given conditions in paper. (A) The hydrolysate chromatograms of EstTB11 and (B) The hydrolysate chromatograms of Palatase 20,000 L.

strates and V_{max} represents the higher catalytic efficiency of lipase [25].

3.8. Analysis of volatile compounds of EstTB11hydrolysates

The two hydrolysates produced by EstTB11 and Palatase 20,000 L were analyzed by SPME-GC/MS. The results showed that 68 volatile compounds in hydrolysates of EstTB11 and 39 volatile compounds in that of Palatase 20,000 L were detected. The volatile components hydrolyzed from EstTB11were much more than those from Palatase 20,000 L, which was consistent with the result of sensory evaluation. 25 volatile compounds were selected to compare from the two hydrolysates as main contributing components in milk flavors improvement.

The chemical name of 25 volatile compounds, CAS number, RI and relative contents are shown in Table 5. As shown in Table 5, EstTB11 and Palatase 20,000 L obvious prefer for short and medium-chain fatty acids, the contents of butanoic acid, hexanoic acid, heptanoic acid and octanoic acid were all increased, but tetradecanoic acid and 9-octadecenoic acid were not change or decreased. The results were consistent with the conclusion of substrate specificity of purified EstTB11. In Table 5, the volatile compounds from EstTB11 (25 °C) had higher contents and quantities than those from Palatase 20,000 L (50 °C), which indicated that low temperature could better keep the flavor substances when the hydrolysis degree of the two enzymes was near to equivalence. Therefore, EstTB11 has a great advantage in enzymolysis temperature and its hydrolysates are better use to produce milk essence seasoning to improve milk flavors. Chromatograms obtained by the SPME-GC/MS are were shown in Fig. 8.

4. Discussion

Microbial esterases/lipases are mostly extracellular, and their production is greatly influenced by the medium compositions, however, an inducer is one of them. The esterase productivity of *Pseudomonas* sp.TB11 was improved to different degrees by induction with adding rapeseed oil, soybean oil, olive oil, single cream and sunflower oil. Therefore, EstTB11 is an induction-type esterase; however, its productivity was sharply inhibited on triolein as an inducer. At present, reports are available regarding the hydrolysis of olive oil [26], butter oil [27], beef tallow [28] and other animal fats [29] using lipase, but data on the use of single cream inducing *Pseudomonas* for the production of esterase have not been reported. In this study, the experiment proved successfully improving EstTB11 activity by induction of *Pseudomonas* sp.TB11 with single cream. This esterase acts preferentially on short-chain fatty acids, which are commonly used to generate desirable flavours in dairy products [12]. The use of single cream as an inducer was found to have a significant correlation with esterase production for strain TB11.

Table 4
Temperature and pH properties of some cold-adapted lipases from different microorganisms.

Microorganism	Optimum temperature(°C)	Relative enzyme activity(temperature)	Optimum pH	References
Pseudomonas sp.TB11(esterase)	25	36% (0 °C)	8.5	This study
Pseudomonas sp.strain B11-1 (esterase)	45	17% (0 °C)	8.0	[35]
Psychrobacter celer 3Pb1(est12)	35	41% (0 °C)	7.5	[33]
Pseudoalteromonas sp. wp27	30	60% (4 °C)	7-8	[37]
Pseudoalteromonas haloplanktis TAC125	40	15% (10 °C)	8.5	[38]
Pseudomonas fragi strain no. IFO3458	29	59% (10 °C)	8	[36]
Pseudomonas sp. KB700A	35	13% (0 °C)	8-8.5	[21]
Pseudomonas sp. 7323	30	20% (0 °C)	9	[7]
Geotrichum sp. SYBC WU-3 (Lipase-A)	20	60% (0 °C)	9.5	[31]
Psychrobacter sp.	20	Not shown	8.0	[8]
Yarrowia lipolytica NCIM 3639	25	40% (5 °C)	5.0	[22]

In previous studies, the available literatures on the purification of cold-adapted esterase were not adequate. A salt-tolerant esterase was purified 42.7-fold with a 6.4% rate of recovery (specific activity, 569.2 U/mg protein) by precipitation with ammonium sulphate followed by anion and cation exchange chromatography [30]. An extracellular lipase from *Y. lipolytica* was purified by Q-Sepharose anion exchange chromatography and Sepharose CL-4B. This resulted in 195 U/mg specific activity and 13.9-fold purification with an 11.14% rate of recovery [22]. Two cold-adapted lipases (Lipase-A and Lipase-B) of mesophilic *Geotrichum* sp. SYBC WU-3 were purified by using $(NH4)_2SO_4$ fractionation, chromatography separation on a DEAE-cellulose-32 column and a Sephadex G100 column. The overall level of purification recovery was 20.4% (11.2% for Lipase-A and 9.2% for Lipase-B) [31]. Compared to these cold-adapted lipases, EstTB11was purified 15.21-fold, with a high specific activity (1526.2 U/mg), using a three-step purification protocol. The purified esterase showed a molecular mass of 65 kDa. In contrast, an esterase (PsEst1) of the psychrotrophic bacterium *Pseudomonas* sp. B11-1 has been found to possess a molecular mass of 69 kDa [32]. A novel esterase Est12 has been reported with an estimated molecular mass of 35.15 kD [33]. The EstA esterase has an apparent molecular mass of about 23 kDa [34]. A lipase from *Pseudomonas stutzeri* PS59 with high detergent performance and cold-adapted possessed a molecular weight of approximately 55 kDa [5]. The molecular mass of the purified esterase in this study is comparatively higher than those reported elsewhere, which indicated that it was a novel enzyme from *Pseudomonas*.

The EstTB11 showed optimal activity at pH 8.5 and was found to have a higher stability over a pH range of 7.0–10.0 at 30 °C. A cold active lipase from *Y. lipolytica* NCIM 3639 showed stability over a pH range of 4–6, with complete loss of activity at pH 7.0 [22]. The *P. stutzeri* PS59 lipase was found to be functionally active in the pH range of 7.0 to 11.0 and displayed optimal activity at pH 8.5 and showed approximately 80% residual activity at pH 9.0 [5]. In addition, the enzyme EstTB11 exhibited maximum activity at 25 °C, and it still retained more than 38% of its maximal activity at 0 °C, comparing with the reported, the relative activity of EstTB11 exhitbited ice-adapted enzyme of extremophiles (Table 4). A list of similar cold-adapted esterases/lipases producing strains are presented in Table 4 to make a comparison with EstTB11. The optimal tempera-

Table 5
25 volatile compounds in the hydrolysates of EstTB11and Palatase 20,000 L identified by SPME-GC/MS.

No.	Compounds	CAS No.	Rical[a]	ID[b]	RI[c]	RT (min)	Relative contents (%)	
							EstTB11	Palatase2000 L
1	Hexanal	66-25-1	1072	RI	1072	7.236	0.122 ± 0.002	0.007 ± 0.003
2	2-Heptanone	110-43-0	1181	RI	1180	10.001	3.572 ± 0.08	0.304 ± 0.007
3	2-Nonanone	821-55-6	1385	RI	1387	15.360	1.862 ± 0.019	0.353 ± 0.011
4	Nonanal	124-19-6	1392	RI	1390	15.440	0.007 ± 0.001	nd
5	1-Octene	111-66-0	1427	MS	–	16.107	0.028 ± 0.004	nd
6	Acetic acid	64-19-7	1449	RI	1448	15.475	0.031 ± 0.004	0.101 ± 0.031
7	Benzaldehyde	100-52-7	1513	RI	1508	17.646	0.007 ± 0.001	nd
8	2-Nonenal, (E)-	18829-56-6	1528	RI	1530	17.876	0.019 ± 0.002	0.003 ± 0.001
9	2-Undecanone	112-12-9	1597	RI	1598	18.798	0.459 ± 0.014	0.27 ± 0.011
10	Butanoic acid	107-92-6	1624	RI	1627	19.130	5.659 ± 0.153	8.573 ± 0.168
11	Pentanoic acid	109-52-4	1732	RI	1736	20.534	0.111 ± 0.043	0.147 ± 0.011
12	2-Dodecanone	6175-49-1	1809	MS	–	21.440	0.131 ± 0.009	0.118 ± 0.178
13	Hexanoic acid	142-62-1	1841	RI	1850	21.784	30.07 ± 1.071	34.32 ± 1.204
14	Heptanoic acid	111-14-8	1953	RI	1954	22.938	1.089 ± 0.066	0.978 ± 0.032
15	Octanoic acid	124-07-2	2061	RI	2060	24.045	30.97 ± 0.444	29.41 ± 0.636
16	Nonanoic acid	112-05-0	2165	RI	2164	25.066	0.311 ± 0.052	0.323 ± 0.029
17	2H-Pyran-2-one, tetrahydro-6-pentyl-	705-86-2	2215	RI	2203	25.499	0.082 ± 0.006	0.071 ± 0.010
18	*n*-Decanoic acid	334-48-5	2273	RI	2279	26.051	17.22 ± 0.866	17.67 ± 0.570
19	9-Decenoic acid	14436-32-9	2336	RI	2335	26.597	1.34 ± 0.004	1.318 ± 0.036
20	Undecanoic acid	112-37-8	2379	RI	2369	26.991	0.104 ± 0.03	0.111 ± 0.05
21	-Dodecalactone	2305-5-7	2393	MS	–	27.094	0.017 ± 0.004	0.014 ± 0.002
22	2H-Pyran-2-one, 6-Heptyltetrahydro-	713-95-1	2440	RI	2458	27.604	0.051 ± 0.006	nd
23	Dodecanoic acid	143-07-7	2487	RI	2490	27.983	1.958 ± 0.128	3.676 ± 0.094
24	Tetradecanoic acid	544-63-8	2713	RI	2714	30.645	0.404 ± 0.056	1.424 ± 0.088
25	9-Octadecenoic acid (Z)-	112-80-1	3180	RI	3173	31.447	0.038 ± 0.008	0.089 ± 0.005

nd: not found.

[a] Linear retention indices calculated of volatile compounds on an DB-WAX capillary column (30 m × 0.25 mm × 0.25 μm) with a homologous series of n-alkanes (C_4–C_{28}).

[b] Identification method: MS, identification by comparing mass spectra in the Wiley7.0 and NIST 2012 library search system; RI, identification by retention indexes with literature data.

[c] From the flavornet database (http://flavornet.org, accessed June 2007), and NIST atomic spectra database.

ture of an esterase PsyEst was found to be around 35 °C [4]. A novel cold-adapted esterase Est12 from a psychrotrophic bacterium *Psychrobacter celer* 3Pb1 displayed the optimal activity at 35 °C and was unstable at temperatures above 40 °C [33]. EstTB11 was quite stable at 15 °C to 35 °C,around 90% of maximum activity at 15–25°C for 720 min and the relative activity at 45 °C remained above 65% for 60 min, which displayed significantly high thermostability. The purified cold-adapted lipase from *Y. lipolytica* NCIM 3639 exhibited maximum activity at 25 °C and retained 40% of its activity at 5 °C. The activity decreased sharply at temperatures above 30 °C. The enzyme was found to be stable at 10 °Cand 30 °C. It lost about 40% of its activity after 6 h of incubation at 35 °Cand was completely inactive at 45 °C after 4 h of incubation [22]. Two new cold-adapted lipases from mesophilic *Geotrichum* sp. SYBC WU-3 have good stability at room temperature, for they can retain 80% of their activity after incubation at 25 °C for 72 and 48 h, respectively [31]. However, some other lipases from psychrotrophic and psychrophilic microorganisms did not show good heat stability. For example, the half-life of lipase from *Pseudomonas* sp. 7323 at 30 °C is only 4.5 h [7]; lipase from *Pseudomonas* sp. B11-1 retains 92% of its activity after incubation at 30 °C for 60 min [35]; half-life of the lipase from *Pseudomonas* fragi is about 5 h at 27 °C [36].

The esterase showed more activity in the presence of K^+, Na^+ and Mg^{2+}, whereas it was inactivated by Ca^{2+}, Al^{3+}, Mn^{2+} and Fe^{3+}. The presence of Fe^{3+} ions significantly inhibited esterase activity at all concentrations, which was consistent with K. Tamilarasan's conclusion [16]. A lipase from *Bacillus sphaericus* MTCC 7542 Mn^{2+} and Na^+ ions in 5 mM and 10 mM enhanced the enzyme activity was observed 10–12% more than the control. Most *Pseudomonas* lipases are inhibited by heavy metal ions such as Zn^{2+}, Hg^{2+}, Cu^{2+}, Ni^+, Cd^+, Fe^{2+}, and Co^{2+}, and are activated by Ca^{2+} and Mg^{2+} [5].

Hydrolysis of milk fat catalyzed by esterase or lipase commonly is applied as a controlled process for the development of desirable flavors in certain products. These applications include flavor development in various cheeses, manufacture of lipolyzed milk fat products for enhancement of butter-like flavors, and flavor development in milk chocolate etc. [39,40]. Flavoring plays a crucial role in the production and research process of milk essence. Moreover, the key components of milk flavor are determined, which is the fundamental and critical step in the work of flavouring. Comparative GC/MS data on EstTB11 and Palatase 20,000 L indicate the hydrolysis degree of the two enzymes was near to equivalence, but low temperature could be better keeping the flavor substances. Therefore, EstTB11 has an advantage in enzymolysis temperature and broader application for flavor development in dairy products, including various cheeses, lipolyzed milk products, bakery, ice cream and flavor preparations, whose potential future applications also are considered.

5. Conclusion

In conclusion we have shown the differential induction of EstTB11 from extremophiles based on single cream as substrate was used for the growth, which could hydrolyze milk fat to produce short and medium-chain fatty acid and this result layed the foundation for the application in increased aroma of milk products. Purification, peptide mass fingerprinting analysis and N-terminal sequencing identification revealed that a novel cold-adapted esterase, EstTB11, was obtained, which enriched the cold active enzyme resource. Biochemical characterisation and application on cream revealed that EstTB11 had advantages in low temperature and broad pH as well as higher thermostability. These indicated that the esterase can be a very attractive enzyme for potential application in biocatalysis.

Acknowledgments

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