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Cloning, Expression, and Characterization of a Novel Thermostable and Alkaline-stable Esterase from *Stenotrophomonas maltophilia* OUC_Est10 Catalytically Active in Organic Solvents

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Abstract: A thermostable and alkaline-stable novel esterase (Est7) was identified through the whole genome sequencing of *Stenotrophomonas maltophilia* OUC_Est10. The open reading frame of this gene encoded 617 amino acid residues. After heterologous expression in *Escherichia coli* BL21 (DE3), the purified Est7 was separated as a single protein and presented a molecular mass of 70.6 kDa. Multiple sequence alignment indicated that Est7 had a typical catalytic triad (Ser-Asp-His) and the conserved sequence (GDSL) typical of the family II lipid hydrolase proteins. Est7 showed good stability in alkaline buffers, especially in Tris-HCl buffer at pH 9.0 (residual activity 93.8% after 96 h at 4 °C) and in the medium temperature conditions (residual activity 70.2% after 96 h at 45 °C and pH 8.0). The enzyme also retained higher stability toward several hydrophilic and hydrophobic organic solvents (e.g., after incubation in 100% acetonitrile or in n-hexane the enzyme retained about 97% and 84% of the activity in the absence of organic solvent, respectively). Furthermore, Est7 could catalyze the transesterification reaction of vinylacetate with 2-phenylethanol and *cis*-3-hexen-1-ol to their corresponding acetate esters in petroleum ether or *tert*-butyl methyl ether. These results indicate Est7 as a promising biocatalyst for applications of Est7 in non-aqueous media.

Keywords: thermostable and alkaline-stable esterase; characterization; transesterification; organic solvents; *Stenotrophomonas maltophilia*

1. Introduction

Esterases (EC 3.1.1.1) can catalyze the hydrolysis or the synthesis of glycerides. Contrary to lipases they have higher substrate specificity toward short chain length glycerides (e.g., carbon chain length <10) [1–4]. Analogously to lipases, apart from the hydrolysis of ester bonds in aqueous systems, esterases can catalyze interesterification, transesterification, esterification, and aminolysis reactions in non-aqueous media (e.g., organic solvents) [5–8].

Esterases usually have high chemical selectivity, regioselectivity, stereospecificity, and stability in organic solvents. They do not need coenzymes and cofactors and are active under mild reaction conditions, which is a prerequisite to obtain fewer by-products in synthetic reactions, thus, making this class of enzymes interesting for organic synthesis purposes at the industrial level [9–11].

With the development of biotechnologies, esterases have been widely used in medicine, food, fine chemicals, and biodiesel industries due to their excellent biological properties [12–14]. However, their use in industrial processes has many restrictions under prolonged catalytic reaction conditions or in the presence of organic solvents. Therefore, the identification and characterization of new enzymes with enhanced thermal and pH stability, and catalytically active and stable in neat organic solvents or in a water–organic solvent mixture, is crucial for industrial applications.

Biocatalysis in organic solvents is a special and practical technique that can increase the number of enzyme applications in organic synthesis, food-related conversions, and analysis [15,16]. In particular, this technique has become the goal of many research activities, allowing to expand the potential of enzymes and develop successful application at the industrial level [17,18]. By changing the organic solvents in the reaction medium, the substrate specificity, the regio- and enantioselectivity of a given enzyme can also be controlled [19–21].

In general, enzymes produced by microorganisms are more widely applicable than those obtained from animals and plants thanks to their lower production cost. Therefore, the increasing demand for novel biocatalysts has led to the development of new methods for screening new genes, such as genome sequencing. Thus, it is possible to obtain more information about lipolytic enzymes sequence by performing whole genome sequencing of known active strains, which is more intuitive and rapid than to screen the lipolytic activity in different strains. *Stenotrophomonas maltophilia* OUC_Est10 is a gram-negative bacterium that has been shown to secrete esterases and lipases [22]. Herein, we aimed at the heterologous expression in *E. coli* of the highly active lipolytic enzymes identified in the whole genome of strain *S. maltophilia* OUC_Est10 [23].

2. Results and Discussion

2.1. Esterase Gene Est7 Sequence and Protein Structure Analysis

Based on the whole genome sequencing of *S. maltophilia* OUC_Est10, the analysis and comparison showed that the genome was rich in lipolytic enzyme genes. The nucleotide sequence of the gene of *est7* has been submitted to the GenBank database with the accession number MH253883.

By phylogenetic analysis, Est7 was assigned to the lipolytic enzyme family II (Figure 1a). This classification was also confirmed through the analysis of the enzyme structure by the SMART website (URL: http://smart.embl.de/), which showed that the sequence from the 1st to the 26th amino acid residue (MLLSKRPIRSLMAAAIALAAVPAMAG) was a signal peptide typically observed for the lipolytic enzyme family II (Figure 1c). Multiple sequence alignment indicated that Est7 had a typical catalytic triad (Ser-Asp-His) and the conserved sequence (GDSL) typical of the family II lipid hydrolase proteins (Figure 1b).

2.2. Heterologous Expression and Purification of Est7

Before heterologous expression in *E. coli*, esterase gene *est7* was inserted into three different plasmids (pET-32a (+), pET-28a (+) or pET-21a (+)). Determination of esterase activity indicated that these three vectors could be used for the expression of Est7. By comparing their enzyme activities, pET-32a (+) was used for further research for its high expression level (data not shown). The purified Est7 was consistent with the predicted molecular mass (70.6 kDa) through sodium dodecyl sulfate polyacrylamide gel electrophoresis sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Figure 2). There was negligible enzyme activity in the pellet of Est7 cell lysate. The protein concentration of purified Est7 and supernatant of centrifuged Est7 cell lysate were 1.29 mg mL^{-1} and 3.36 mg mL^{-1}, respectively. The yield of purified Est7 per mL of bacterial culture was 0.29 mg, and the specific activity of purified Est7 toward *p*-nitrophenyl butyrate was 69 U mg^{-1}.

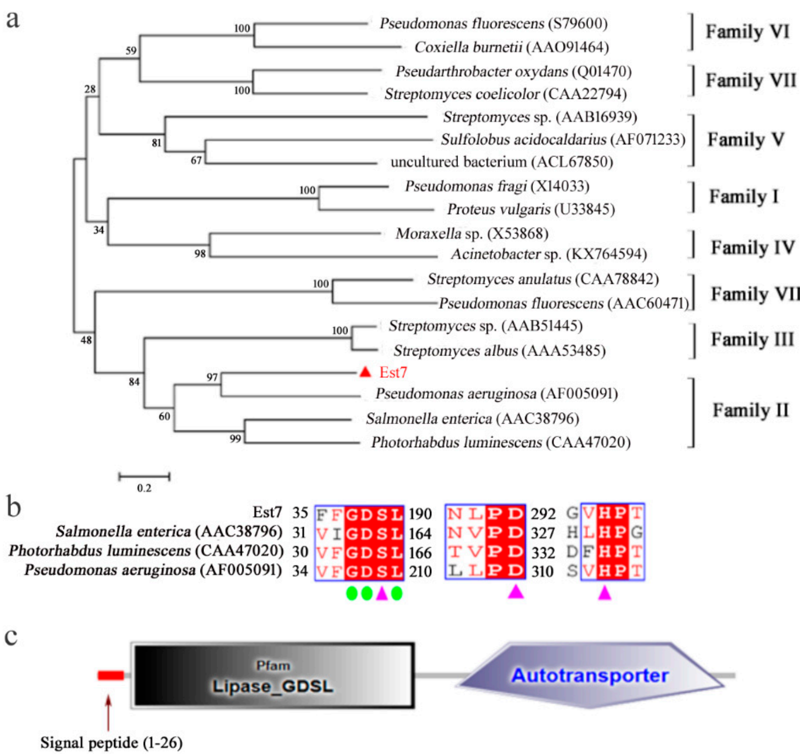

Figure 1. Bioinformatic analysis of Est7. (**a**) Neighbor-joining phylogenetic tree. Est7 is shown as red triangles. (**b**) Multiple sequence alignments of Est7 and other lipolytic enzymes belonging to the family II. The typical motif is indicated using green circles, and the catalytic triad (Ser, Asp, His) is emphasized with pink triangles. (**c**) Analysis of Est7 protein structure.

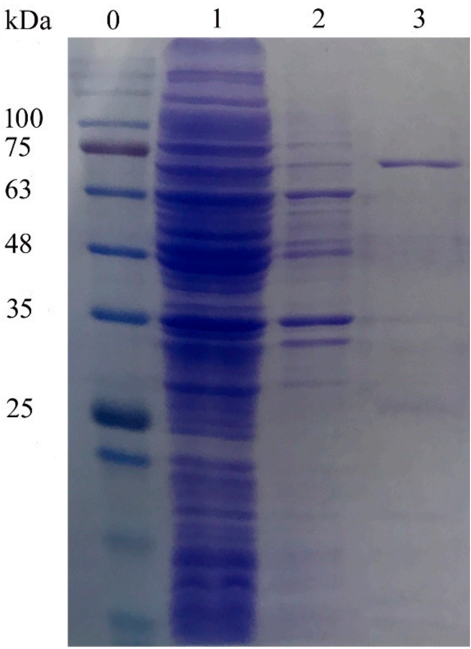

Figure 2. SDS-PAGE analysis of Est7. Lane 0, marker; Lane 1, supernatant of centrifuged Est7 cell lysate; Lane 2, pellet of Est7 cell lysate; Lane 3, purified Est7.

2.3. Enzyme Characterization of Purified Esterase Est7

Substrate preference for Est7 was determined with *p*NP esters with different acyl chain lengths. In Figure 3a it can be observed that Est7 had a relatively high activity with esters with carbon chain

length <10. In particular, Est7 displayed the highest activity toward *p*NP caproate (*p*NPC6), and the lowest activity was observed for *p*NP palmitate (*p*NPC16). This result indicates that Est7 is an esterase that preferentially catalyzed the hydrolysis of short carbon chain length [11,24].

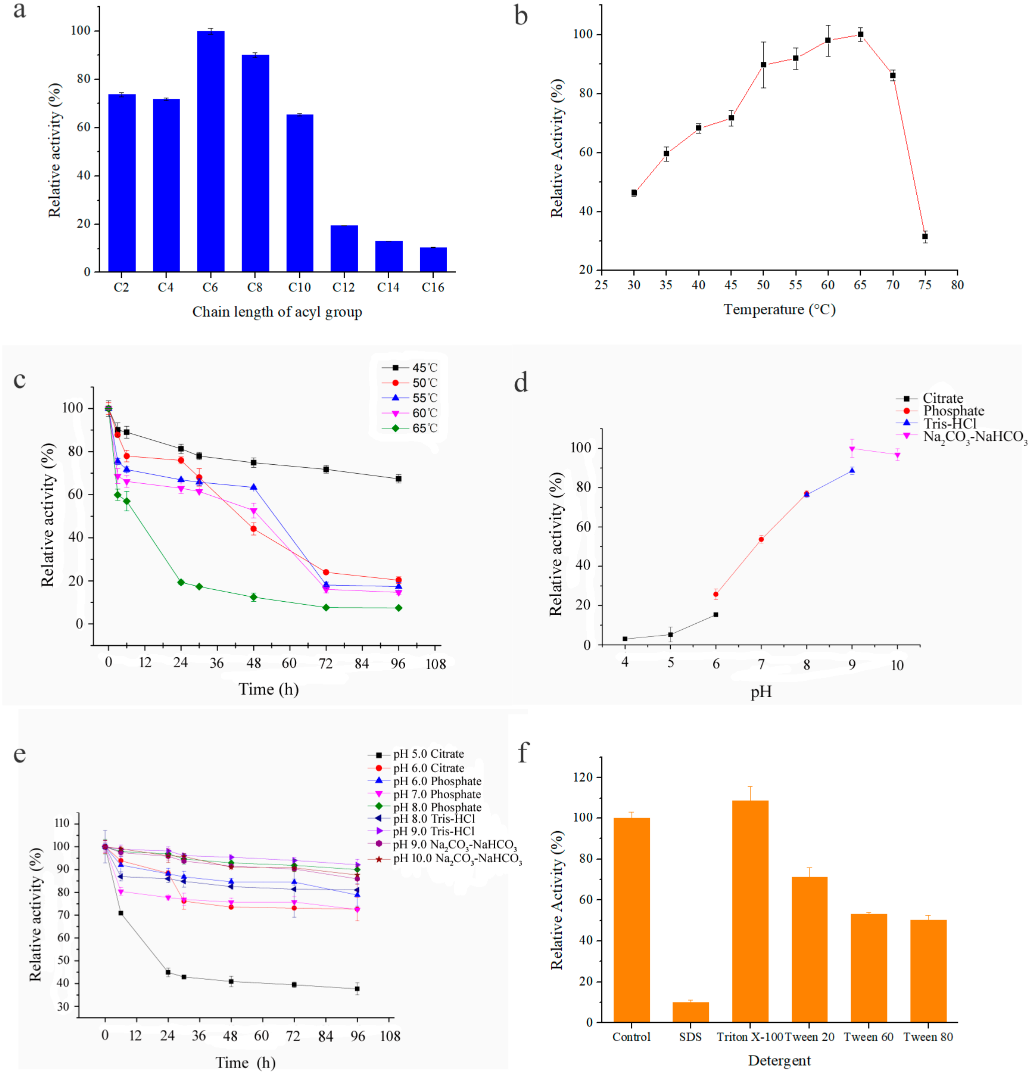

Figure 3. Characterization of Est7. Substrate specificity of Est7 on various *p*NP esters (**a**). Effect of temperature on Est7 activity (**b**). Residual activity of Est7 after incubation in 0.1 M Tris-HCl buffer, pH 8.0, at 45, 50, 55, 60, or 65 °C for various durations (**c**). Effect of pH on Est7 stability. The buffers used were citrate (■), phosphate (●), Tris-HCl (▲), and Na_2CO_3–$NaHCO_3$ (▼). The activity value obtained at pH 9, was considered 100% (**d**). Relative activity of Est7, measured after incubation up to 96 h in different 0.1 M buffers and at different pHs. The activity at time 0 for the different pH values was considered 100% (**e**). Effects of surfactants (0.5%) on Est7 activity, the reaction without surfactant addition (control) was defined as 100% (**f**). Except when differently specified *p*-NP butyrate was used as the substrate (see Materials and Methods, Section 3.4 for experimental details).

Concerning the effect of temperature on Est7 activity, the activity of Est7 increased up to 65 °C and then declined (Figure 3b). Interestingly, the optimal temperature for the activity of this enzyme was higher than we found for Est3-14 (60 °C), recently isolated and purified [25]. Therefore, Est7 might be more suitable than this latter enzyme for applications at high-temperature conditions.

The activity of Est7 after incubation at 45 °C for 96 hours was 70.2% of that of the control. At 55 °C and 60 °C, after 48 hours of incubation, the enzyme retained more than 60% of its original activity. However, the residual activity decreased to 20% of the original activity within 24 h at 65 °C

(Figure 3c). Based on these data, Est7 appears a mesophilic enzyme that maintains activity at medium and high temperatures.

As shown in Figure 3d, Est7 had relatively high enzyme activities under alkaline conditions and was pH-sensitive, with an optimal activity at pH 9.0 in Na_2CO_3-$NaHCO_3$ buffer and inactivation at pH < 5.0.

The pH stability of the Est7 was determined by measuring the residual activity after incubation for different times (4 °C, up to 96 h) in different pH value buffers (pH 5.0–10.0). As shown in Figure 3e the residual activity of Est7 after 96 h in Tris-HCl buffer, pH 9.0, only slightly decreased (93.6% of the initial activity). This result was similar to that obtained with esterase Est3-14 (residual activity 94%, 4 °C, pH 8.0, and 36 h) reported by Lu et al. [25]. Furthermore, at pH 8.0 and 10.0, the residual activity of Est7 was higher than 85%, indicating that Est7 had good alkali resistance, analogously to EST4 (40 °C) described by Gao et al. [8]. However, in acidic conditions, beyond citrate buffer at pH 5.0 where the residual enzyme activity decreased to about 40% of its initial activity, the residual enzyme activity of Est7 was higher than 70% at pH 6.0, indicating a good stability also at acidic pH values.

Different surfactants (tested at 0.5% *v*/*v* concentration) were added to the reaction system to study their effect on the esterase activity of Est7. As shown in Figure 3f, Triton X-100 had a slight promotion effect on enzyme activity (114.8%). Tween 80, Tween 60, Tween 20, and SDS all had an inhibitory effect on enzyme activity. SDS, an anionic surfactant, could destroy the non-covalent bonds between enzyme molecules, and change the conformation of the enzyme. It had a strong inhibitory effect on the esterase activity of Est7.

The effect of metal ions on the activity of Est7 was determined by adding different concentrations of metal ions to the reaction system. The results are shown in Table 1. K^+ and Ca^{2+} had a promoting role on the esterase activity of Est7, especially at when used at 1 mM concentration. In particular, the addition of 1 mM K^+ increased enzyme activity up to 150% of the activity in the absence of metal ions. On the contrary, the addition of 10 mM Cu^{2+}, Mg^{2+}, Fe^{3+} or Co^{2+} decreased the activity 15 to 45% (the inhibitory effect was most pronounced with Fe^{3+} and enzyme activity decreased to 55% of the control activity). The addition 1 mM of Cu^{2+}, Ni^{2+}, Zn^{2+}, Mg^{2+}, Fe^{3+} or ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) had little effect on the esterase activity of Est7.

Table 1. Effect of Na_2-EDTA and metal ions on Est7 activity.

Ion	Relative Activity (%) [a]	
	1 mM	10 mM
Control	100 ± 2.8	100 ± 1.2
Na_2-EDTA	96.7 ± 1.5	85.3 ± 2.1
Co^{2+}	78.1 ± 1.7	67.6 ± 2.8
K^+	150.2 ± 2.1	112.2 ± 2.8
Fe^{3+}	92.3 ± 3.2	55.1 ± 2.8
Ca^{2+}	121.3 ± 1.8	109.2 ± 1.7
Mg^{2+}	98.7 ± 2.3	76.5 ± 2.7
Zn^{2+}	98.5 ± 2.3	87.5 ± 1.2
Ni^{2+}	95.7 ± 1.4	85.1 ± 0.6
Cu^{2+}	93.8 ± 2.4	56.6 ± 2.7

[a] The catalytic activity toward *p*NPB without any metal ions was taken as 100%. All measurements were repeated three times.

Organic solvents are usually added to the reaction medium in biocatalyzed reaction to facilitate the solubilization and hydrolysis of hydrophobic substrates. However, the addition of organic solvent can affect enzyme activity and the final yield of the reaction. In particular, water-miscible organic solvents generally deprived the enzyme of the water molecules necessary for its structure and activity, resulting in the inactivation of the enzyme. Therefore, the effect of different organic solvents on Est7 activity was examined [26], and results are shown in Table 2. At a concentration of organic solvent

of 25% (v/v), Est7 maintained good activity (>70% of the initial value) in all cases tested. With the increase of concentration to 50% (v/v), the enzyme activity of Est7 showed a declining trend, but when the concentration of hydrophilic organic solvent was increased to 100% (v/v) (neat organic solvent), the enzyme activity of Est7 improved. After incubation in pure acetonitrile solvent, the enzyme activity of Est7 was 97.2% of that just solubilized in buffer, without any organic solvent (control), whereas the residual enzyme activity was 64.7% after incubation in 50% (v/v) acetonitrile solvent. This result could be explained considering that in water–organic solvents mixtures protein structural unfolding can occur more easily than in neat organic solvents, as observed with different enzymes [27–29]. For the same reason and similar to other hydrolases, Est7 was stable also in hydrophobic organic solvents. After incubation in 100% n-hexane, Est7 retained 83.8% of the activity in the absence of organic solvent, which indicated that Est7 had good stability in both hydrophilic and hydrophobic organic solvents. In particular, compared with other reports, Est7 showed higher stability than rEstSL3 [30] and LipBA45 [31] in organic solvents. These features are good prerequisites for the application of this enzyme in biocatalytic reactions.

Table 2. Effects of organic solvents on Est7 activity.

Organic Solvents	**log *P* [a]**	**Residual Activity (%) [b]**		
		25 [c]	**50 [c]**	**100 [c]**
Control		100 ± 3.4	100 ± 2.1	100 ± 3.2
DMSO	−1.3	76.6 ± 1.8	56.9 ± 2.7	32.1 ± 0.9
Methanol	−0.76	93.6 ± 4.3	70.7 ± 1.2	38.8 ± 1.7
Ethanol	−0.24	92.38 ± 3.5	71.3 ± 0.8	55.3 ± 4.1
Acetone	−0.23	88.4 ± 1.9	66.9 ± 3.2	80.5 ± 3.3
Acetonitrile	−0.15	90.2 ± 0.3	64.7 ± 2.1	97.2 ± 0.2
Isopropyl alcohol	0.1	94.5 ± 0.6	78.2 ± 1.2	41.2 ± 0.4
n-Propyl alcohol	0.28	97.6 ± 0.4	86.3 ± 0.7	50.8 ± 1.5
Chloroform [d]	2.0	70.5 ± 1.7	60.5 ± 1.7	24.3 ± 0.8
Cyclohexane [d]	3.2	89.2 ± 1.4	77.8 ± 1.4	87.4 ± 1.6
n-Hexane [d]	3.5	75.3 ± 2.4	66.7 ± 2.1	83.8 ± 1.8
Isooctane [d]	4.5	74.7 ± 2.5	62.1 ± 2.3	73.4 ± 2.6

[a] Log *P* value is the partition coefficient of an organic solvent between water and *n*-octanol phases. [b] After pretreating Est7 for 3 h in different organic solvents, the remaining enzymatic activity was measured in Tris-HCl buffer (100 mM, pH 8.0) by using *p*NPB as the test substrate. An enzyme sample incubated in buffer only was used as the measure of 100 % activity. [c] Organic solvent concentration (%, *v/v*) in the enzyme solution. [d] The percentage of organic solvent is intended as the percentage in the biphasic organic solvent/water system.

2.4. Catalytic Activity of Esterase Est7 in Organic Solvents

To further expand the applications of esterase Est7, this enzyme was tested in the transesterification reaction of vinyl acetate with different alcohols (substrate 2-phenylethanol or *cis*-3-hexen-1-ol) and carried out in pure organic solvents (petroleum ether or *tert*-butyl methyl ether) as the reaction medium. As shown in Figure 4, comparing the results of Gas chromatography (GC) analysis of the reaction mixture without enzymes and the standard of products, it could be found that Est7 displayed high transesterification activity in the non-aqueous systems, allowing the synthesis of *cis*-3-hexen-1-yl acetate and 2-phenethyl acetate. Furthermore, quantitative analysis of products was carried out by internal standard method. Under the reaction conditions employed, the concentration of 2-phenethyl acetate and *cis*-3-hexen-1-yl acetate was 0.192 M and 0.189 M, respectively, after 12 h in *tert*-butyl methyl ether. The presence in the chromatogram of all the reaction samples of peaks at around 6.1 and 6.7 min (Figure 4e–h) could be due to the presence of impurities (or their derivatives caused by the reaction with vinyl acetate) present in the enzyme preparation or in the molecular sieves, the extra peaks having the same retention times, independently of the alcohol or of the organic solvent tested. However, these impurities do not interfere with the formation of the acetyl esters, which indicates that Est7 is exploitable in applied biocatalysis in non-aqueous media.

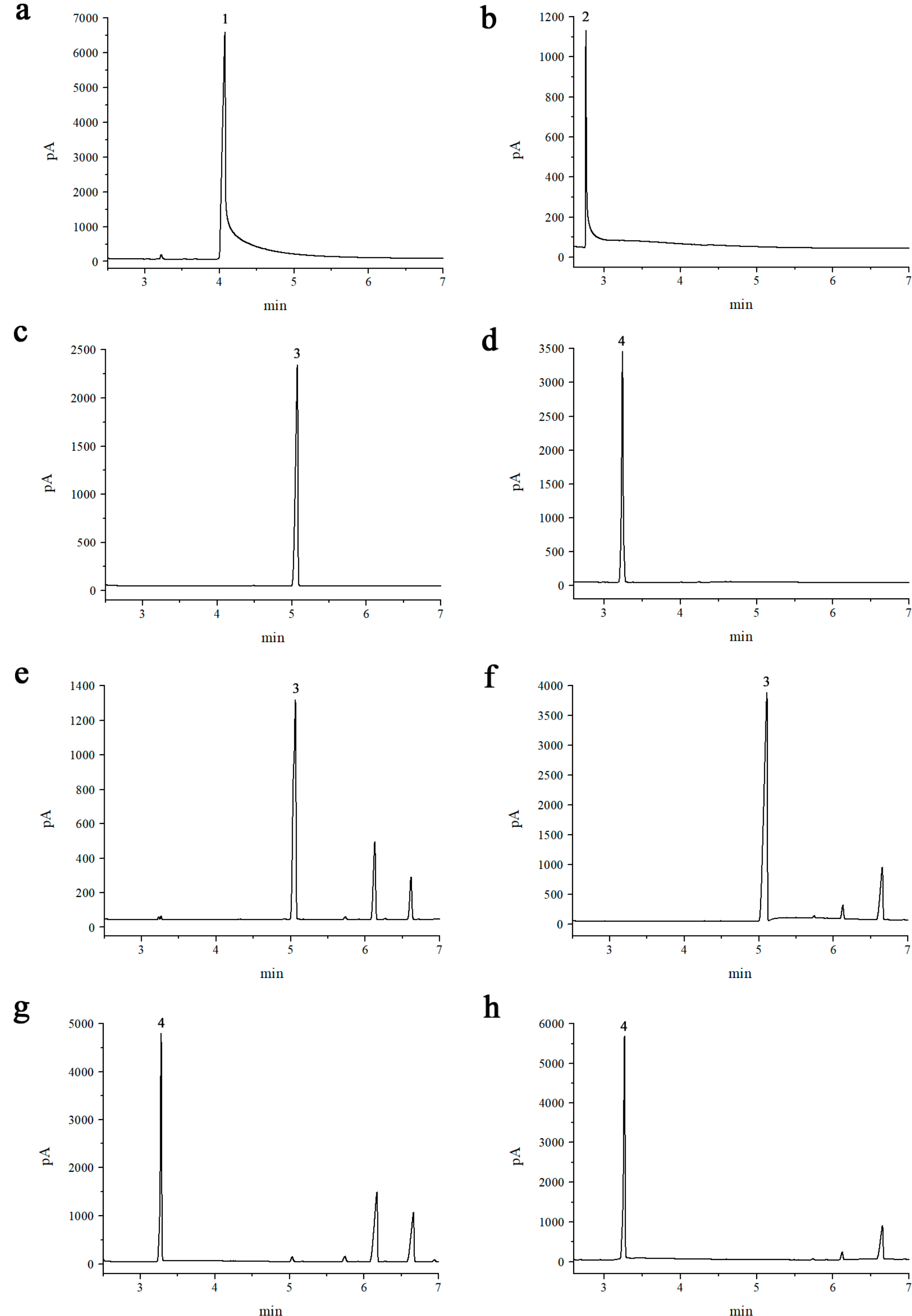

Figure 4. Comparing the results of GC analysis of the reaction medium without Est7 (**a**,**b**) and the standard of product (2-phenethyl acetate, *cis*-3-hexen-1-yl acetate) (**c**,**d**). GC analysis of the transesterification between 2-phenylethanol and vinylacetate to give 2-phenethyl acetate and acetaldehyde in petroleum ether (**e**) or *tert*-butyl methyl ether (**f**) and of the transesterification between *cis*-3-hexen-1-ol and vinylacetate to give *cis*-3-hexen-1-yl acetate and acetaldehyde in petroleum ether (**g**) or *tert*-butyl methyl ether (**h**). Peak 1, 2-phenylethanol (retention time 4.1); Peak 2, *cis*-3-hexen-1-ol (retention time 2.8); Peak 3, 2-phenethyl acetate (retention time 5.1); Peak 4, *cis*-3-hexen-1-yl acetate (retention time 3.3). In all cases, the reaction time was 12 h. The chromatographic analysis of a sample prepared by mixing volumes (1:1) of the reaction sample and of the standard sample of the expected ester produced a chromatogram with only one peak at the retention time corresponding to that of the ester (data not shown).

3. Materials and Methods

3.1. Materials

Restriction enzymes (*Bam*HI, *Eco*RI, and *Hin*dIII) and T4 DNA ligase were obtained from Thermo Fisher Scientific (US). *E. coli* DH5α and BL21 (DE3) competent cells were from Tiangen Biochemical Technology Co., Ltd. (Beijing, China). Vinyl acetate (purity percentage ≥99.5%) was obtained from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). The *p*-nitrophenol (*p*NP) and its esters were purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular sieves were purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China) and all other reagents used were of analytical grade unless otherwise specific instructions.

3.2. Bacterial Strains, Plasmids, and Sequence Analysis of Lipolytic Genes

The complete genome sequence of *S. maltophilia* OUC_Est10 consists of a single chromosome of 4,668,743-bp with a CG content of 66.25% [23]. Sixteen genes corresponding to the predicted lipolytic enzymes were selected for cloning and expression. Among them, the esterase gene *est7* ligated to pET-32a (+) had hydrolytic activity on the *p*NP esters of C4-C16, suggesting its potential for use in the synthesis of long-chain fatty acid glycerides. The nucleotide sequence of esterase gene *est7* sequence information was analyzed by the OUC_Est10 full genome annotation information [23]. The length of the esterase gene *est7* sequence was 1854 bp.

Phylogenetic tree of esterase Est7 and other lipid hydrolase families was analyzed by MEGA 6.0 software according to the classification reported by Arpigny and Jaeger [32]. The expression plasmids used were select pET-28a (+), pET-21a (+), and pET-32a (+), and the recombinant vectors were transformed into *E. coli* BL21 (DE3) to express the active protein.

3.3. Heterologous Expression and Purification of Esterase Est7

To heterologously express an esterase Est7 in *E. coli*, the constructed primers according to the nucleotide sequence of the Est7 were (*Eco*RI) 5′-CCGGAATTCATGCTGCTCAGCAAACGCC-3′ (forward) and Reverse (*Hin*dIII) 5′-CCCAAGCTTTTAGAAGCTGCCGCTGAAGTTG-3′ (Reverse), where the underlining indicated the restriction sites. After digestion with *Eco*RI and *Hin*dIII, the fragment amplified by PCR was inserted into pET-32a (+), and the recombinant vector was transformed into *E. coli* BL21 (DE3) for expression. The recombinant strain was grown in lysogeny broth (LB) medium, and cultured in a shaker at 37 °C until the OD_{600} reached 0.6 to 0.8, and then isopropyl-*β*-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce expression at 20 °C for 20 h (200 rpm). After 20 h, the fermentations were centrifuged at 4 °C for 20 min (5439× *g*), then cells were harvested and disrupted in Tris-HCl buffer (pH 8.0, 100 mM) (buffer A) by ultrasonic fragmentation (ultrasound 3 s, interval 5 s, power 200–400 W, 100 cycles). The cell-free extracts were prepared by centrifugation at 4 °C for 20 min (5439× *g*), the precipitate was discarded.

The purity of Est7 and the expression level was assayed by SDS-PAGE standard method [33]. The suspension was sonicated, and the supernatant was loaded on a Ni-nitrilotriacetic acid (Ni-NTA) column (1 mL, Qiagen, Hilden, Germany). The Est7 was eluted by gradient elution with buffer A containing different concentrations (20 mM–500 mM) of imidazole.

3.4. Enzyme Characterization of Purified Esterase Est7

The esterase Est7 activity was assayed by a spectrophotometric method using *p*-nitrophenyl butyrate (*p*NPB) as a substrate and determining the *p*-nitrophenol (*p*NP) produced at 405 nm. To this end, 20 μL of enzyme solution (1.29 mg mL^{-1}) was added to 0.75 mL of Tris-HCl (100 mM, pH 8.0) buffer containing 20 μL of *p*NPB substrate solution (20 mM *p*NPB dissolved in isopropyl alcohol and dimethyl sulfoxide (*v*/*v*, 3/1)) and the mixture placed in a 37 °C water bath for 5 min. The reaction was stopped adding SDS (final concentration 1%), and the absorbance was measured at 405 nm. An esterase

activity unit (U) is defined as the amount of enzyme required to produce 1 μmol of *p*NP per minute. Soluble protein content was estimated by the Bradford assay [34].

The substrate specificity of the purified enzyme Est7 was tested by using *p*NP esters containing different carbon chain length (*p*NP acetate (C2, *p*NPC2), *p*NP butyrate (C4, *p*NPC4), *p*NP caproate (C6, *p*NPC6), *p*NP octanoate (C8, *p*NPC8), *p*NP decanoate (C10, *p*NPC10), *p*NP laurate (C12, *p*NPC12), *p*NP myristate (C14, *p*NPC14), and *p*NP palmitate (C16, *p*NPC16).

Effect of temperature on esterase Est7 activity was studied in the range of 30 to 75 °C, in buffer A and with *p*NPB as the substrate. The enzyme activity at the optimum temperature was defined as 100%, and the activity at other temperatures was expressed as a percentage of the highest activity. The thermostability of Est7 was tested by incubating the enzyme in buffer A at 45 °C, 50 °C, 55 °C, 60 °C, or 65 °C for 96 h, respectively.

Effect of pH on enzyme activity of Est7 was studied using different buffer solutions with different pH values. The buffers used were: 100 mM citrate buffer for pH 4.0–6.0, 100 mM phosphate buffer for pH 6.0–8.0, 100 mM Tris-HCl buffer solution for pH 8.0–9.0, 100 mM Na_2CO_3–$NaHCO_3$ buffer for pH 9.0–10.0. To determine the pH stability of Est7, the purified Est7 was incubated for 36 h in different buffers.

Effect of surfactants on the enzyme activity of Est7 activity was evaluated by adding different surfactants (Tween 20, Tween 60, Tween 80, and SDS) to a final concentration of 0.5% in buffer A. The enzyme without the addition of a surfactant was used as the control.

The effect of metal ions on the activity of esterase Est7 was to add metal ions (Co^{2+}, K^+, Fe^{3+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+} or Cu^{2+}) and Na_2-EDTA to the buffer A at a final concentration of 1 mM and 10 mM. The enzyme activity of the sample in which metal ions and Na_2-EDTA were not added was defined as 100%.

The effect of organic solvents on Est7 enzyme activity was studied mixing dimethyl sulfoxide (DMSO), methanol, ethanol, acetone, acetonitrile, isopropyl alcohol, n-propyl alcohol, chloroform, cyclohexane, n-hexane or isooctane with the enzyme solution (pH 8.0, 100 mM Tris-HCl buffer solution, 1.29 mg mL^{-1}) at a final concentration of 25%, 50% or 100%. The mixture was shaken at 30 °C for 3 h, and then the residual enzyme activity was determined as described above. In the case of hydrophobic organic solvents, the biphasic system (organic solvent/enzyme solution) after the incubation was centrifuged to remove the organic solvent, and the enzyme activity of Est7 was measured under standard conditions. In the case of hydrophilic organic solvent, the mixed solution after the incubation was diluted with buffer A until the concentration of the organic solvent was 5% to reduce the effect of remaining organic solvents on enzyme activity determination.

3.5. Esterase Est7 Catalyzed Transesterification

To study the application of esterase Est7 in synthetic reactions in neat organic solvents, the purified enzyme Est7 was previously frozen at −80 °C and then lyophilized. The transesterification between 2-phenylethanol (*cis*-3-hexen-1-ol) and vinylacetate to give 2-phenethyl acetate (*cis*-3-hexen-1-yl acetate) and acetaldehyde was used as the model reaction. For the transesterification reaction, 360 U of lyophilized biocatalyst (69 U mg^{-1}) was added to 1 mL of organic solvent (petroleum ether or *tert*-butyl methyl ether) containing 0.2 M alcohol (substrate 2-phenylethanol or *cis*-3-hexen-1-ol) and 0.6 M vinyl acetate and adding molecular sieves (80 mg of molecular sieve per mL of reaction) to ensure dry conditions. The reaction mixture was incubated at 37 °C for 12 h in a rotary shaker (180 rpm). At predetermined timepoints, aliquots were withdrawn from the reaction mixtures and the conversion determined by GC analysis. The reaction mixtures without Est7 was used as the control. A 6890 gas chromatograph (Agilent Technologies, USA) equipped with a flame ionization detector (FID) was used. The stationary phase was an HP-5 capillary column (30 m × 250 μm × 0.25 μm). The mobile phase was nitrogen, and the oven temperature was varied from 120 °C (initial time 0.5 min) to 180 °C with a heating rate of 10 °C min^{-1} [20].

The internal standard method was used to quantify the reaction product using n-octanol (2 μL·mL^{-1} in *tert*-butyl methyl ether) as the internal standard. The internal standard solution was used to prepare a 0.1 M 2-phenethyl acetate or *cis*-3-hexen-1-yl acetate (standard) solution, and the peak area/moles ratio for the internal standard (A_s/m_s) and for the ester (A_r/m_r) were determined and used to calculate the ratio.

$$f = \frac{\frac{As}{ms}}{\frac{Ar}{mr}} \tag{1}$$

Then the reaction solution containing the internal standard (2 μL·mL^{-1}) was injected, and the concentration of products was calculated according to the peak response value of the internal standard as follow:

$$mi = f \times \frac{Ai}{\frac{As}{ms}} \tag{2}$$

where A_i is the peak area of the product and A_s/m_s is the peak area/moles ratio for the internal standard determined in the analysis of the reaction sample.

4. Conclusions

The identification herein presented of a moderately thermophylic (optimum temperature at 65 °C) and alkaline-stable novel esterase (Est7), and confirmed *S. maltophilia* OUC_Est10 as a source of numerous lipolytic and esterolytic activities. The isolated enzyme also showed a good tolerance toward both hydrophobic and hydrophilic organic solvents. These properties, and the fact that Est7 could catalyze transesterification reaction in pure organic solvents, highlight the exploitability of this enzyme in numerous biotechnological applications including in organic synthesis reactions. Furthermore, the possibility to overexpress Est7 in *E. coli* BL21 (DE3) made this enzyme particularly interesting for large scale applications. In addition, further improvements of stability and specificity can also be envisaged by enzyme engineering of Est7. Finally, the approach herein followed for the identification of new enzyme activities (e.g., searching new enzyme activity from the whole genome sequence of known active strains) resulted as very promising for the identification of new efficient and versatile enzymes and a more rapid method than screening new enzymes from different strains.

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