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Facile One-Pot Immobilization of a Novel Esterase and Its Application in Cinnamyl Acetate Synthesis

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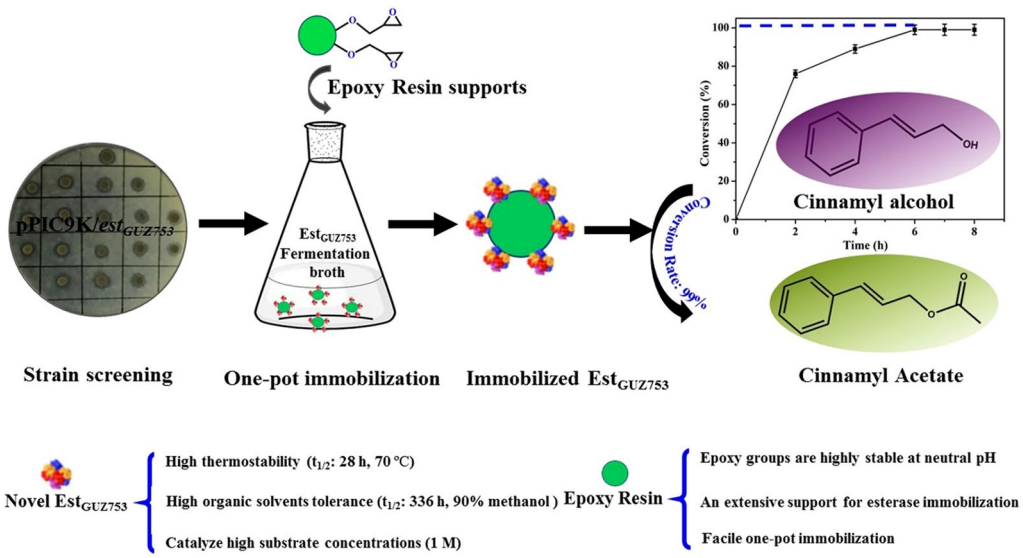
Abstract

Cinnamyl acetate has a wide application in food industries. This work focuses on cinnamyl acetate synthesis with a novel esterase. est_{GUZ753}, an esterase gene from *Geobacillus uzenensis* DSMZ 13551, was first cloned and expressed in *Pichia pastoris* KM71. The optimal activity of Est_{GUZ753} towards *p*-NP caprylate was at pH 8.0 and 70 °C, and the half-life at 70 °C was 28 h. Furthermore, Est_{GUZ753} showed marked tolerance in methanol. The activity of Est_{GUZ753} increased up to 1.16-fold in 90% methanol after 72 h, and the half-life in it was 336 h. The crude fermentation broth of Est_{GUZ753} was immobilized directly onto the epoxy resin (Lx-105s), and the immobilized Est_{GUZ753} exhibited a 99% conversion for cinnamyl acetate synthesis at a high cinnamyl alcohol concentration in 6 h (cinnamyl alcohol: 1.0 M, enzyme dosage: 3%). These characteristics of Est_{GUZ753} indicated its great potential for organic synthesis.

Graphic Abstract

A novel esterase gene from *Geobacillus uzenensis* DSMZ 13551 was first cloned and expressed in *Pichia pastoris* KM71. Esterase Est_{GUZ753} showed marked thermostability at high temperature and tolerance in methanol. Furthermore, the crude fermentation broth of Est_{GUZ753} was immobilized directly onto the epoxy resin, and immobilized Est_{GUZ753} exhibited a 99% conversion for cinnamyl acetate synthesis.



Keywords High thermostability · High organic solvents tolerance · Esterase · Transesterification · Cinnamyl acetate

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1 Introduction

Cinnamyl acetate is one of the most important flavor esters. It has a wide application in the food, pharmaceuticals and cosmetic industries due to its sweet, balsamic and floral odor [1]. Moreover, cinnamyl acetate has many beneficial biological properties, such as antioxidant activity, which is ascribed to its special structural features [2]. Traditionally, cinnamyl acetate was synthesized by chemical synthesis of an alcohol with an organic acid in the presence of strong acids. But it was not low-cost input or environmentally friendly approach. Chemical synthesis produced undesirable, non-specific, toxic byproducts and harsh conditions to carry out the reactions [3, 4]. Thus, recently, enzyme catalyzed transesterification and esterification are attractive alternatives to avoid these drawbacks, which are the most economical approaches to reach the green products with no harm to human health [5].

Current researchers are targeting lipase-mediated catalysis reaction for flavor esters [6–9]. Sharma et al. obtained the maximum conversion was only 55% after 27 h with porcine pancreatic lipase [10]. Yadav et al. used Novozym 435 to synthesize ethyl cinnamate with 60% conversion in 2 h at 30 °C [11], Wolfson et al. obtained a high yield of ethyl cinnamate at room temperature, whereas it took 96 h to obtain it [12]. The mostly studies are reported at a low concentration or conversion of cinnamyl alcohol using lipase. One reason may be that most enzymes exhibited an undesired activity and stability in organic solvents, especially in polar organic solvents [13]. In order to meet the industrial demands, many esterases have been engineered to enhance their thermostability by site directed mutagenesis. However, site-directed mutations mostly leaded to a decrease in activity. Thus the wild-type thermostable esterase with high activity and organic solvents tolerance has much commercial value. In addition, in order to increase recyclability and economics of those excellent enzymes, various esterases expressed in *Escherichia coli* (*E. coli*) had been immobilized onto epoxy-functionalized supports, which was very stable at neutral pH and wet conditions for a long time [14–16]. However, the esterase expressed in *E. coli* needed to be isolated from *E. coli,* which resulted in an unsatisfactory decreased activity. To overcome this major drawback, the enzyme expressed in *Pichia pastoris* KM71 was fully released into the medium, which behaved like a purified enzyme, thereby obviating the need for cost-intensive isolation, purification, and could be immobilized directly on some supports via one-pot strategy.

In this study, a novel esterase gene, est_{GUZ753} from *Geobacillus uzenensis* DSMZ 13551 was successfully expressed *P. pastoris*. Furthermore, Est_{GUZ753} fermentation broth was directly immobilized on epoxy-functionalized supports via one-pot strategy. The immobilized Est_{GUZ753} exhibited a high conversion for cinnamyl acetate synthesis towards cinnamyl alcohol with a high concentration. The enzyme dosage, substrate concentrations, reaction time and temperature were invested.

2 Materials and Methods

2.1 Materials, Strains and Plasmids

Epoxy resin Lx-105s was provided by Xian Lanxiao Technology New Materials Co., Ltd. (Xian, China). *G. uzenensis* DSMZ13551 was preserved in our laboratory. *P. pastoris* strain KM71 was grown in YPD broth (2% dextrose, 2% tryptone, 1% yeast extract) at 30 °C, or MD broth (2% dextrose, 4×10^{-5}% biotin, 1.34% YNB and 2% agar) or BMMY broth (1% yeast extract, 1.34% YNB, 4×10^{-5}% biotin, 2% dextrose, 100 mM potassium phosphate buffer, 0.5% methanol) at 30 °C. The plasmid pPIC9K (Invitrogen, Carlsbad, USA) was the vector used to construct the protein expression. The substrate *p*-NP esters were purchased from Sigma (St. Louis, MO, USA). DNA polymerase and restriction enzymes were purchased from Takara (Kyoto, Japan). Genomic DNA was isolated using genomic DNA isolation kit (Vitagene, China).

2.2 Construction and Expression of Est_{GUZ753} in *P. pastoris*

Using the primers GUZ753-U/GUZ753-D (Table S1), the PCR product was cloned into pPIC9K by digested with *Eco*R I. To enable gene integration, the recombinant plasmid was linearized with *Sal* I enzyme and purified. Then, it was transformed into the expression strain *P. pastoris* KM71 by the Gene Pulser Xcell™ Electroporation System (Bio-Rad, Hercules, CA, USA). The conditions were 1.5 kV, 200 Ω, 25 μF, 5 ms followed by pulsing. The recombinant strains were grown at 30 °C on MD agar plates until single yeast colonies appeared. The single yeast colonies were identified with two pairs of primers, AOX sequencing primers, and primers GUZ_{753}-U/GUZ_{753}-D (Table S1). Subsequently, colonies with large hydrolyzing halos were selected and grown in BMMY broth for 5–7 days, and the expression of Est_{GUZ753} was induced by methanol with 0.5% (v/v) every 12 h.

2.3 Biochemical Characterization of Est_{GUZ753}

Enzyme activities of Est_{GUZ753} solution were assayed by measuring the absorbance at 405 nm of liberated *p*-nitrophenol. One unit of enzymatic activity was defined as the amount of enzyme needed to release 1 µM *p*-nitrophenol per minute. We detected the substrate specificity using the following substrates: *p*NP-acetate (C2), *p*NP-butyrate (C4), *p*NP-caproate (C6), *p*NP-caprylate (C8), *p*NP-decenoate (C10), *p*NP-laurate (C12), *p*NP-myristate (C14), and *p*NP-palmitate (C16). The enzyme assay was carried out under standard assay conditions.

The pH optimum for the enzyme activity was studied over a range from pH 4–9 for 5 min (60 °C). The following buffer systems were used: pH 4.0–5.0 with 100 mM citric acid-sodium citrate, pH 6.0–7.0 with 200 mM sodium phosphate, pH 8.0 with 50 mM Tris–HCl, and 9.0 with 50 mM glycine–NaOH. The effect of temperature was assayed at 30–90 °C (pH 8.0). The thermostability of Est_{GUZ753} was evaluated by assaying its residual activity after incubation at various temperatures (50–90 °C) for a certain time. Aliquots were removed at various intervals and the residual esterase activity was determined. Non-incubated enzyme was determined as the 100% activity value.

Half-life ($t_{1/2}$) of Est_{GUZ753} in methanol (50%, 60%, 70%, 80% and 90%, v/v) was calculated by measuring the residual activities after different incubation times as described by Li et al. [17]. The Est_{GUZ753} was mixed with different volumes of methanol solution to a final volume of 1 mL and incubated for periods at 4 °C (200 rpm). (For example, a 60% methanol solution contained 100 µL Est_{GUZ753} solution, 300 µL Est_{GUZ753} buffer, and 600 µL methanol). Then 20 µL aliquots was removed from the mixture and transferred into 960 µL esterase buffer with 20 µL substrates, and residual activities were determined under standard conditions. For other organic solvent tolerance of the Est_{GUZ753}, aliquots of the recombinant Est_{GUZ753} solution were respectively incubated in 20%, 50% and 90% (v/v) organic solvents, such as toluene, cyclohexane, *n*-hexane, *n*-heptane and isooctane, After 24 h treatment, the residual activity was measured as described above.

The effects of metal ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Fe^{2+}) on the activity of Est_{GUZ753} were determined at final concentrations of 1, 5 and 10 mM.

2.4 Sequence Analysis

The nucleotide sequences and predicted amino acid sequences were analyzed by the BLAST program (NCBI). ClustalX (version 1.83) was employed to analyze multiple sequence alignments [18]. The SWISS MODEL server (https://swissmodel.expasy.org/SWISS-MODEL.html) was adopted for predicting its three-dimensional structure. The model was visualized by PDB Viewer and the figure was constructed by Pymol.

2.5 Immobilization of the Est_{GUZ753} on Epoxy Resin (Lx-105s) and Enzymatic Synthesis of Cinnamyl Acetate

The yeast cells were centrifuged at 7000×*g* for 10 min at 4 °C and the supernatants was passed through a 0.22 µm filter to remove large particles of impurities. Then the solution was concentrated (5 folds) using a 50 mL Amicon Ultra Centrifugal Filter Device with a molecular weight cut-off of 10 kDa (Millipore, USA). The hydrolytic activity of Est_{GUZ753} was 410 U/mg according to the method of Wang et al. [19]. Because a fivefold concentrated supernatant of Est_{GUZ753} still had a high purity, the supernatant of Est_{GUZ753} was immobilized directly on supports and the procedure was as follows: 10 g epoxy resin (Lx-105s) was equilibrated with 100 mM phosphate buffer (pH 8.0) in a conical flask (200 mL) and stirred at 30 °C for 1 h (200 rpm). Subsequently, 1 g of wet supports and different amounts of Est_{GUZ753} suspension mixed with equal volume of phosphate buffer were added to the 250 mL conical flask, which was stirred in a thermostatic air bath shaker at 30 °C for 7 h (200 rpm). After immobilization, the suspension was filtered and the resins were washed with buffer until no protein was detected in the eluate. Finally, the immobilized Est_{GUZ753} was dried in a vacuum desiccator at room temperature for 10 h and stored at 4 °C until use [20].

The cinnamyl acetate was synthesized through the transesterification using the immobilized Est_{GUZ753} as catalyst. The reaction was carried out in a 50 mL flat bottom glass reactor. The entire reactor was immersed in an ultrasonic thermostatic water bath, which was maintained at desired temperature. The reaction parameters were as follows:

2.5.1 Effect of Reaction Solvent

Different organic solvents not only affect the solubility of hydrophobic substrates but also the activity of the enzyme. Thus the medium toluene, cyclohexane, *n*-hexane, *n*-heptane and isooctane were tested, respectively.

2.5.2 Effect of Molar Ratio

The addition of cinnamyl alcohol (1 M) remained unchanged. The molar ratio of alcohol and vinyl acetate was 1:1, 1:2, 1:3, 1:4 and 1:5.

2.5.3 Effect of Reaction Temperature

The effect of reaction temperature was studied at 20 °C, 30 °C, 40 °C, 50 °C, 60 °C and 70 °C, and other conditions stayed unchanged.

2.5.4 The Effect of Water Content

Other conditions stayed unchanged. Different water content (0.5%, 1%, 5%, 10%, 20% and 50% (w/w, with respect to total substrates)) was analyzed.

2.5.5 Effect of Reaction Time

Other reaction conditions were under the optimum. The effect of reaction time was 2, 4, 5, 6 and 7 h.

2.5.6 Effect of Enzyme Dosag

Different amounts of immobilized Est_{GUZ753} was investigated under the optimal conditions (*N*-hexane as the solvent, molar ratio = 1:4, temperature = 60 °C, water content was 0.5%). The enzyme dosage was as follows: 1%, 2%, 3%, 4%.

The conversion of cinnamyl alcohol is represented by the reduction rate of cinnamyl alcohol in the HPLC chromatograms:

$$\text{conversion ratio} = 1 - A_t/A_0 \quad (1)$$

where A_0 is the area cinnamyl alcohol at time 0 and A_t is the area cinnamyl alcohol at time t.

3 Results and Discussion

3.1 Gene Cloning and Sequence Analysis of Est_{GUZ753}

The esterase gene est_{GUZ753} from the *G. uzenensis* DSMZ 13551 was amplified by primers GUZ753-U/GUZ753-D. The nucleotide sequence had been deposited in the GenBank under the accession number KY612321.

Multiple sequence alignment (Fig. 1a) analysis indicated Est_{GUZ753} was a member of α/β hydrolase family. Est_{GUZ753} contained a single catalytic triad (Ser97, Asp196, and His226) and the consensus pentapeptide G-x-S-x-G, which further revealed that Est_{GUZ753} was a typical member of α/β hydrolase family. As Est_{GUZ753} had not been previously

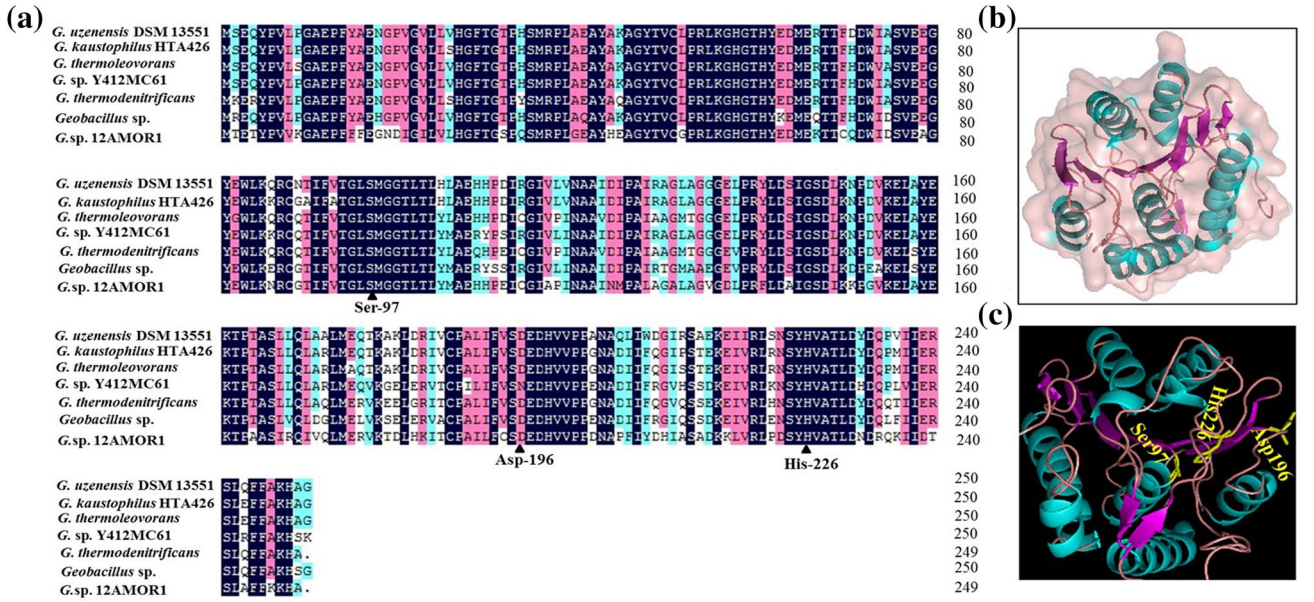


Fig. 1 Protein sequence and structure of Est_{GUZ753}. **a** Sequence alignment of the conserved region of Est_{GUZ753} and other alpha/beta fold hydrolases from different bacterial sources. The alignment strains and identities were as follows: *G. kaustophilus* HTA426 (identity: 93%); *G. thermoleovorans* (identity: 88%); *Geobacillus* sp. Y412MC61 (identity: 86%); *G. thermodenitrificans* (identity: 85%); *G. genomosp.* 3 (identity: 80%); *Geobacillus* sp. 12AMOR1 (identity: 67%); filled triangle, the catalytic site (Ser97, Asp196, and His226); **b** The whole three-dimensional structure. The three-dimensional structure of Est_{GUZ753} was predicted by the SWISS-MODEL server; **c** The catalytic site of Est_{GUZ753} structure. The catalytic triad: Ser97, Asp196, and His226 residues are marked in yellow

reported, the three-dimensional structure of Est_{GUZ753} was generated with a specific template (PDB code: 4LHE (89.6% identity)) (Fig. 1b), which was a lipase from *Bacillus* sp. (strain H-257), and the protein structure was viewed by PDB Viewer. The catalytic triad was observed in the regions (Fig. 1c).

3.2 Heterologous Expression of Est_{GUZ753}

The est_{GUZ753} without signal peptide coding sequence was inserted into the pPIC9K vector and then transformed into the *P. pastoris* KM71. The single colony with a big and clear hydrolysis halo in tributyrin plate was selected to grow in BMMY broth (Fig. 2a). Upon the induction of methanol after six days, the supernatant of the fermentation broth was concentrated by fivefold and analyzed by SDS-PAGE (Fig. 2b). The results showed that the Est_{GUZ753} had a high expression and the molecular mass was about 27 kDa, which coincided with the actual value (27.48 kDa).

3.3 Enzymatic Properties of Free Est_{GUZ753} and Immobilized Est_{GUZ753}

The esterase activities toward various *p*-NP esters were examined at 60 °C and pH 8.0 using recombinant Est_{GUZ753}. Results showed that C8 was the most favorable substrate, and the specific activity of Est_{GUZ753} towards C8 was 205 U/mL (410 U/mg) of protein (Fig. 3a).

The effect of pH on free Est_{GUZ753} activity was determined using C8 as substrate at various pH. The Est_{GUZ753} exhibited higher activities over a pH range of 6.0–8.0, among which the highest specific enzyme activity was at pH 8.0. The activity of Est_{GUZ753} decreased significantly more than pH 8.0 and only about 10% of the maximal activity at pH 9.0 (Fig. 3b). In addition, compared with free Est_{GUZ753}, immobilized Est_{GUZ753} exhibited a wider pH rage (Fig. S1a).

The effect of temperature on free Est_{GUZ753} activity was shown in Fig. 3c. Free Est_{GUZ753} displayed activity over a broad range from 30 to 90 °C, especially between 60 and 80 °C, it retained at least 70% of its maximal activity. Est_{GUZ753} exhibited the maximal activity at 70 °C. In addition, compared with free Est_{GUZ753}, immobilized Est_{GUZ753} exhibited a wider temperature rage (Fig. S1b).

The thermostability of free Est_{GUZ753} was shown in Fig. 3d. The $t_{1/2}$ of Est_{GUZ753} at 70 °C was 28 h, furthermore, over 50% of its maximal activity remained after incubation at 80 °C for 2 h. Kumar et al. reported the best mutant (LipR5) displayed the $t_{1/2}$ was only 2 h at 65 °C. Acharya et al. obtained the mutants, $t_{1/2}$ of TM with significant increase in residual activity was only 1 h at 55 °C [21, 22]. Compared with the above mutants, the results suggested that wild-type Est_{GUZ753} had a broad temperature range and excellent thermostability in high temperature. In addition, about the thermostability, a comprehensive comparison of esterases from different sources at various temperatures was shown in Table 1. The comparison results indicated that recombinant Est_{GUZ753} was more thermotolerant than that of most reported esterases. Furthermore, compared with free Est_{GUZ753}, the $t_{1/2}$ of immobilized Est_{GUZ753} at 70 °C increased up to 47 h, which revealed that immobilization strategy further increased the thermostability of Est_{GUZ753} (Fig. S2).

To estimate comprehensively the potential use of Est_{GUZ753} in organic synthesis reactions, both hydrolytic and hydrophobic solvents were studied. Figure 4 showed that hydrophilic solvents, methanol noticeably activated the activity of Est_{GUZ753}. The highest activity of Est_{GUZ753} increased up to 1.55 and 1.32 folds than the initial activity

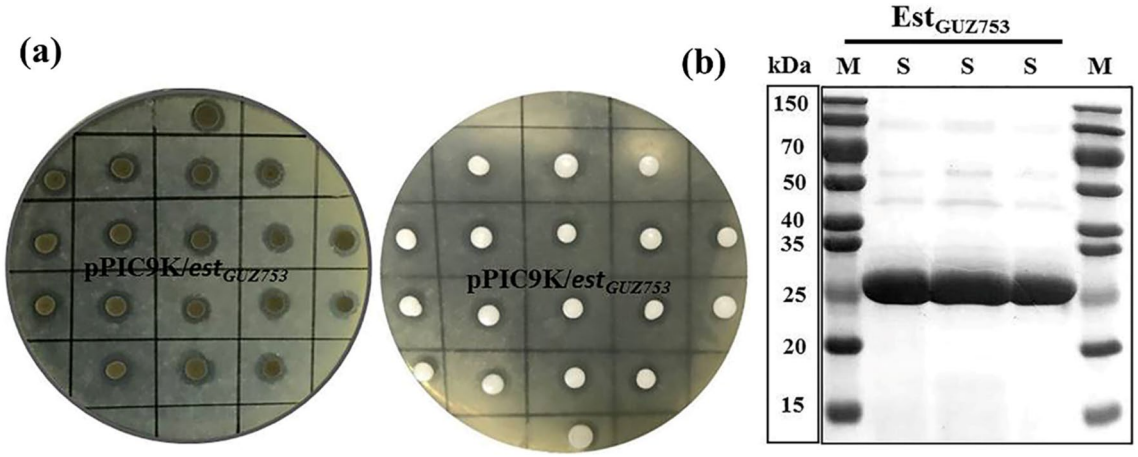


Fig. 2 Enzyme activity identification and SDS-PAGE analysis of protein expression. **a** Hydrolysis halos formed by different clones in tributyrin plates, which indicated that recombinant *P. pastoris* cells were with enzyme activity; **b** The SDS-PAGE analysis of recombinant protein Est_{GUZ753}. Lanes: M, protein marker; S, supernatant of the Est_{GUZ753} fermentation broth

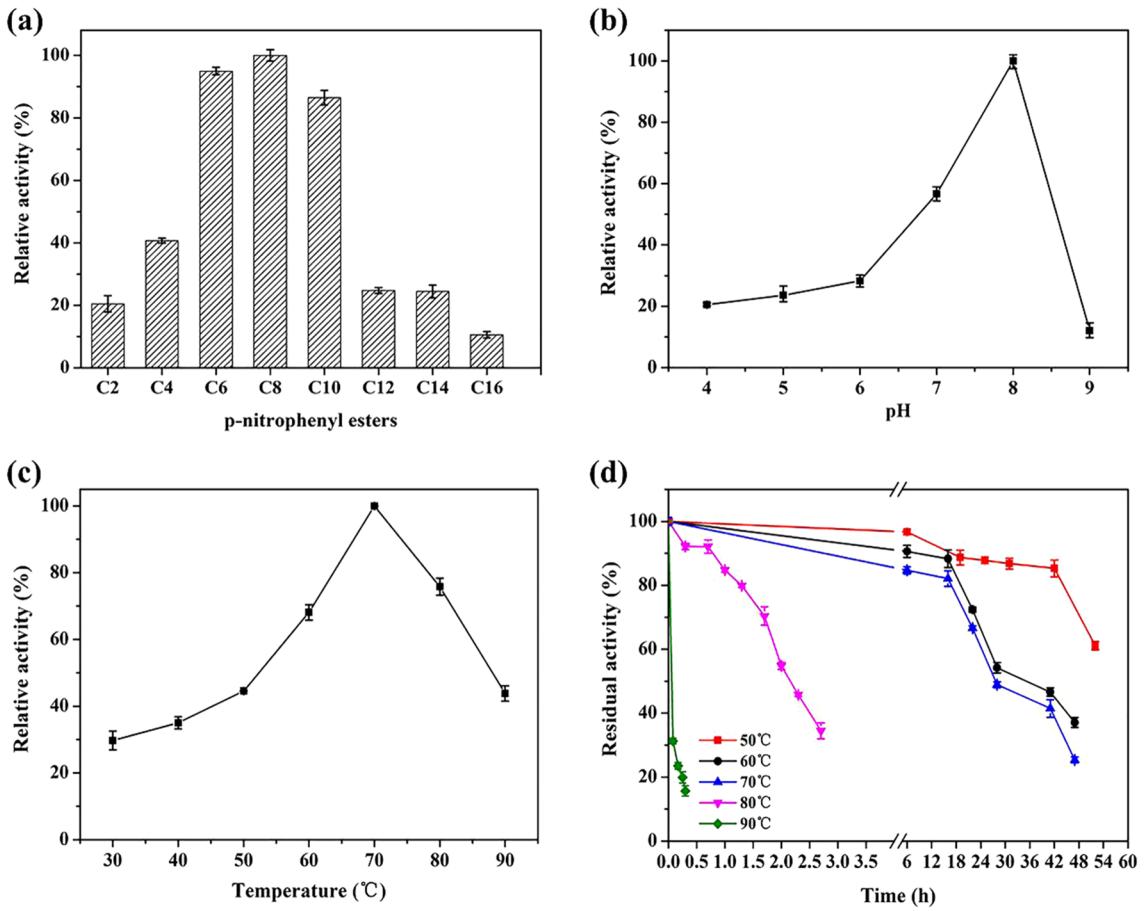


Fig. 3 Enzymatic properties of free Est_{GUZ753}. **a** Substrate specifity of Est_{GUZ753} using *p*NP esters with different acyl chain lengths (C2, C4, C6, C8, C10, C12, C14, and C16); **b** Effect of pH on Est_{GUZ753} activity in different buffers using C8 as substrate; **c** Optimal temperature of Est_{GUZ753} at temperatures ranging from 30 to 90 °C; **d** Thermostability of Est_{GUZ753} at temperatures ranging from 50 to 90 °C. Thermal denaturing half-lives of the recombinant Est_{GUZ753} were determined by measuring residual activities after the enzyme was treated at 50, 60, 70, 80 and 90 °C for a period of time, each value represented the mean and SD of triplicate experiments

Table 1 Comparison of the thermostability of esterases from different sources at different temperatures

Enzyme	Source	T_{opt} (°C)	Thermal stability at various temperatures				
			50 °C	60 °C	70 °C	80 °C	90 °C
Est_{GUZ753}	*G. uzenensis* DSMZ 13551 (this study)	70	61%, 52 h	54%, 28 h	49%, 28 h	55%, 2 h	20%, 15 min
ThLip2	*Thermoanaerobacterium thermosaccharolyticum* [23]	75	–	–	–	50%, 2 h	–
Est7	*Stenotrophomonas maltophilia* OUC_Est10 [24]	60	45%, 48 h	–	Unstable	Unstable	Unstable
GthFAE	*Geobacillus thermoglucosidasius* DSM 2542T [25]	50	64%, 48 h	–	–	–	–
Aaeo1	*Aquifex aeolicus* VF5 [26]	80	–	75%, 2 h	–	–	–
Est-gela	*Bacillus gelatini* KACC 12197 [27]	65	–	–	59%, 1 h	0%, 30 min	Unstable
Esterase	*Bacillus licheniformis* [28]	45	–	70%, 1 h	20%, 1 h	Unstable	Unstable
Esterase	*Bacillus circulans* [29]	60	–	–	100%, 1 h	80%, 1 h	0%, 1 h

in 50% and 60% methanol after 96 h, and in 70%, 80% and 90% methanol, the highest activity of Est_{GUZ753} respectively increased up to 1.26, 1.23 and 1.16 folds than the initial activity after 72 h. Increasing the incubation time, the methanol with different concentrations displayed an inhibitor effect on Est_{GUZ753} activity, the $t_{1/2}$ values of Est_{GUZ753} in 50%, 60%, 70%, 80% and 90% methanol were 34,560, 23,040, 21,600, 20,400 and 20,160 min, respectively. For the high methanol-tolerant lipase, Dror et al. had the similar studies on lipase T6, but $t_{1/2}$ values of the best variants (T6:

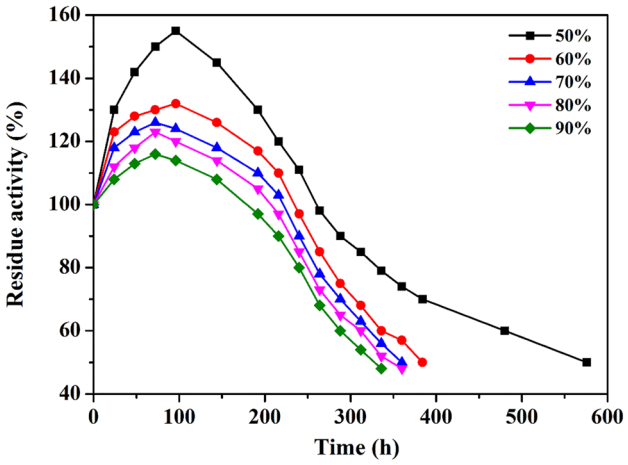


Fig. 4 The relative activity profiles of free Est_{GUZ753} after different incubation time in 50%, 60%, 70%, 80% and 90% methanol (v/v). The activity of Est_{GUZ753} under the standard activity assay conditions (50 mM Tris–HCl (pH 8) buffer, C8 as substrate) was measured after different incubation times in different alcohol concentrations. The residual activity was calculated by comparing the activity of each Est_{GUZ753} before and after incubation in alcohol. The activity in the Tris–HCl buffer without methanol at 70 °C was set as 100%. Each value represented mean and SD of triplicate experiments

H86Y/A269T) were respectively 2429, 1777 and 258 min in 50%, 60% and 70% methanol (Table 2) [30]. There are no reports of esterases showing so high tolerance in 80% and 90% methanol. For the activation phenomenon of methanol with suitable concentration for a period of time, similar effects of methanol on several lipases had been reported, however, some previous studies were in a low concentration of methanol, Cao et al. reported that lipaseTt from *Thermus thermophilus* HB8 exhibited an activation phenomenon in methanol, however, only in 45% methanol [31]. Yamashiro et al. also reported that a cold-adapted and organic solvent-tolerant lipase from *Pseudomonas* sp. Strain YY31 was very active and stable in methanol, whereas it was only 1.02 and 1.10 folds than the initial activity in only 25% and 50% methanol [32]. There are no reports of activation in so high concentration of methanol.

As shown in Table 3, Est_{GUZ753} was also stable in hydrophobic organic solvents (log $P \geq 2.0$) from 20 to 90%. In 90% hydrophobic organic solvents, the Est_{GUZ753} all retained above 85% of its original activity after incubation for 24 h. Furthermore, Est_{GUZ753} retained over 98% of activity in

Table 3 Effect of hydrophobic organic solvents on Est_{GUZ753} activity

Organic solvents	log*P*[a]	Residual activity (%)[b]		
		20%	50%	90%
Control	–	100 ± 1.0	100 ± 0.9	100 ± 1.2
Toluene	2.5	62 ± 1.8	78 ± 1.4	85 ± 1.8
Cyclohexane	3.2	75 ± 1.6	82 ± 2.5	90 ± 1.2
N-hexane	3.5	80.8 ± 3.8	91.4 ± 2.6	98.7 ± 2.7
N-heptane	4.0	71.7 ± 2.9	88.8 ± 1.5	95.5 ± 2.7
Isooctane	4.5	55.1 ± 1.7	71.1 ± 2.7	90.1 ± 1.5

[a]log*P* value is the partition coefficient of an organic solvent between water and *n*-octanol phases

[b]After incubating Est_{GUZ753} for 24 h in different organic solvents, the residual enzymatic activity was measured in 50 mM Tris–HCl buffer (pH 8.0) at 70 °C using C8 as the test substrate. The enzyme sample incubated in buffer only was set as 100%. The presented results were the average of three repeated experiments with SD of ± 5%

N-hexane after 24 h. Generally, many transesterification and ester synthesis reactions are in hydrophobic organic solvents medium, such as *n*-hexane, therefore, the remarkable stability of Est_{GUZ753} makes it an attractive candidate for transesterification reactions [33, 34].

As shown in Table 4, the activity of Est_{GUZ753} was stimulated with the addition of Mg^{2+} and Ca^{2+} at 1 mM, 5 mM and10 mM, and the activity of Est_{GUZ753} increased up to 1.25-fold by adding 1 mM Mg^{2+}. Whereas the activity of Est_{GUZ753} was inhibited by the addition of Mn^{2+}, Zn^{2+}, Co^{2+}, $Ni^{2+}$and Fe^{2+} at different concentrations. Furthermore, Zn^{2+} was an important inhibitor, the addition of Zn^{2+} (10 mM), Est_{GUZ753} nearly lost all the activity.

3.4 Immobilization of Est_{GUZ753} and Its Application in the Synthesis of Cinnamyl Acetate

The immobilization process is outlined in Scheme 1a. The effects of the initial dosage of Est_{GUZ753}, immobilization time on the loading capacity, and the relative esterase activity were investigated (not shown). The final results showed that the highest loading of Est_{GUZ753} was 55.5 mg/g when 90 mg Est_{GUZ753} and 1 g wet supports were initially added in the system, and the immobilized yield was 61.7% (Table S2).

To obtain a higher conversion at a high substrate concentration, the immobilized Est_{GUZ753}-mediated transesterification with cinnamyl alcohol and vinyl acetate was carried

Table 2 Comparison of the half-life between T6 and Est_{GUZ753}

Items	$t_{1/2}$ (min)				
	50% methanol	60% methanol	70% methanol	80% methanol	90% methanol
Est_{GUZ753}	34,560	23,040	21,600	20,400	20,160
lipase T6	347	173	4	–	–
T6: H86Y/A269T	2429	1777	258	–	–

Table 4 Effect of various metal ions on Est_{GUZ753}

Ion	Residual activity (%)[a]		
	(1 mM)	(5 mM)	(10 mM)
None	100 ± 0	100 ± 0	100 ± 0
Mg^{2+}	125.3 ± 2.1	105.1 ± 1.9	103.3 ± 1.9
Ca^{2+}	95.7 ± 1.8	105.7 ± 2.1	103.2 ± 2.2
Mn^{2+}	89.7 ± 3.2	81.1 ± 3.6	48.8 ± 2.5
Zn^{2+}	59.1 ± 3.7	23.5 ± 2.7	ND
Co^{2+}	97.5 ± 2.9	89.3 ± 3.5	80.4 ± 2.4
Ni^{2+}	96.1 ± 2.3	92.9 ± 2.4	88.4 ± 2.0
Fe^{2+}	86.5 ± 3.4	64.9 ± 2.6	41.4 ± 1.7

[a]The esterase was pre-incubated with the metal ions for 1 h at 4 °C before measuring the residual activity. The presented results were the average of three repeated experiments with SD of ± 5%. The activity toward C8 without any metal ions was taken as 100%

ND not detectable

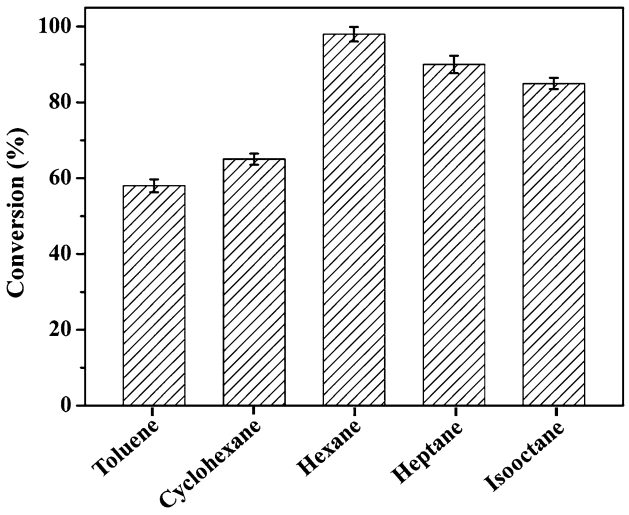
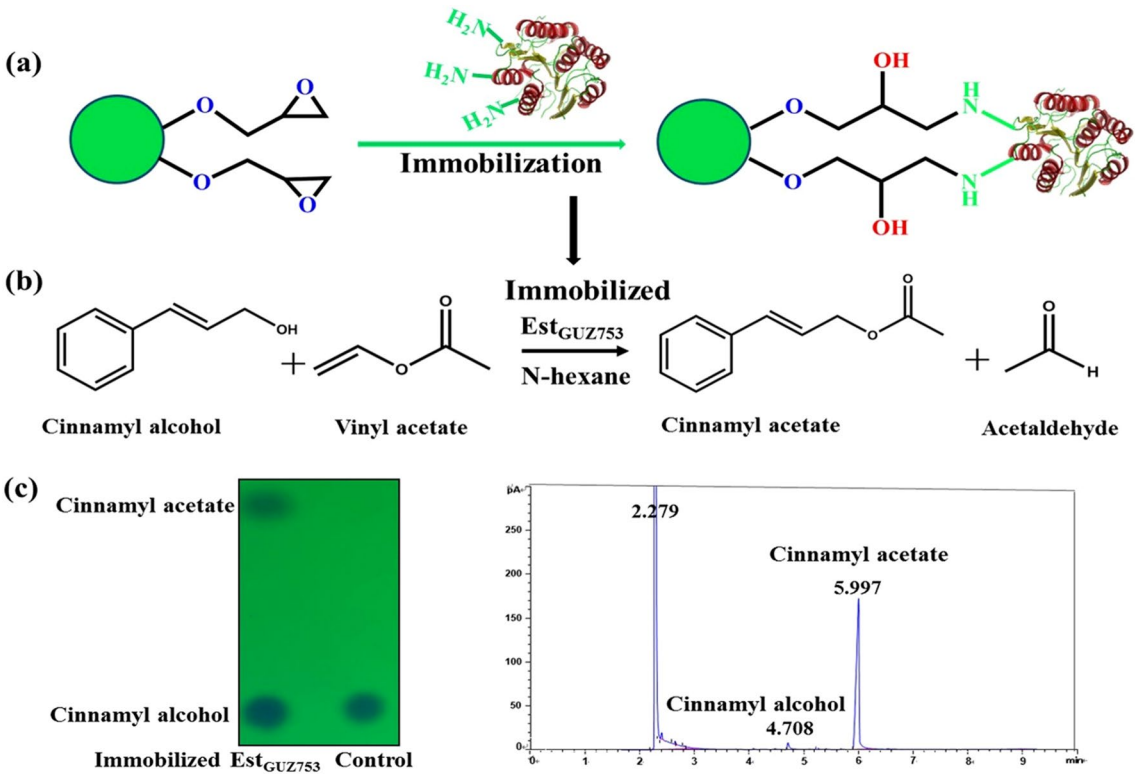


Fig. 5 Effect of hydrophobic organic solvents on cinnamyl acetate synthesis. The maximal activity was defined as 100% and the relative activity is shown as a percentage of maximal activity

out in *n*-hexane (Scheme 1b) and products were analyzed by thin-layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC) (Scheme 1c). For cinnamyl acetate synthesis, Fig. S3 showed that the low concentration (< 1 M) of cinnamyl alcohol could be fully converted to cinnamyl acetate in less than 5 h, however, when the concentration increased up to 1 M, only 85% of cinnamyl alcohol was converted to cinnamyl acetate, so 1 M



Scheme 1 Immobilized Est_{GUZ753}-mediated transesterification of cinnamyl alcohol and vinyl acetate in *n*-hexane. **a** The immobilization procedure of Est_{GUZ753} on Epoxy Resin (Lx-105s); **b** Preparation of cinnamyl acetate through transesterification with immobilized Est_{GUZ753}; **c** TLC and HPLC analysis of cinnamyl alcohol and cinnamyl acetate

of cinnamyl alcohol in this experiment was used to be optimized. Because reaction solvent not only affected the conversion but also affected the enzyme activity by changing the three-dimensional conformation of proteins [7]. The effects of toluene, cyclohexane, *n*-hexane, *n*-heptane and isooctane were shown in Fig. 5, cinnamyl alcohol had a high conversion in different hydrophobic organic solvents, and the highest conversion was obtained in *n*-hexane. The reason may be that hydrophobic organic solvents do not strip the critical water from the enzyme surface, so esterase will retain a high enzyme retention activity in *n*-hexane [35, 36].

The effect of the molar ratio of the two substrates was investigated by TLC and HPLC under the concentration of cinnamyl alcohol at 1 M for 6 h (60 °C) (Fig. 6a). The conversion of cinnamyl alcohol was increased with the molar ratio from 1:1 to 1:4, and the highest conversion was at 1:4.

The effect of temperature was shown in Fig. 6b. The results showed that the conversion of cinnamyl alcohol increased from 30 to 95% in 6 h with the temperature from 20 to 60 °C, however, an obvious decrease in the conversion (79%) occurred at 70 °C. This is possible that the higher temperature had a negative impact on the three-dimensional structure of the enzyme, which reduced the enzyme activity. Therefore, 60 ºC was suitable for transesterification.

Water content in organic bio-synthesis was essential for enzyme flexibility and catalytic activity [37]. The effect of water content on conversion was examined from 0.5% to 50% (w/w). As shown in Fig. 6c, under the addition of 0.5% water, the conversion of cinnamyl alcohol had an obvious increase compared to without water (Fig. 6b) at 60 °C for 6 h. But when the water content increased up to 50%, only 10% conversion was obtained. This result was consistent

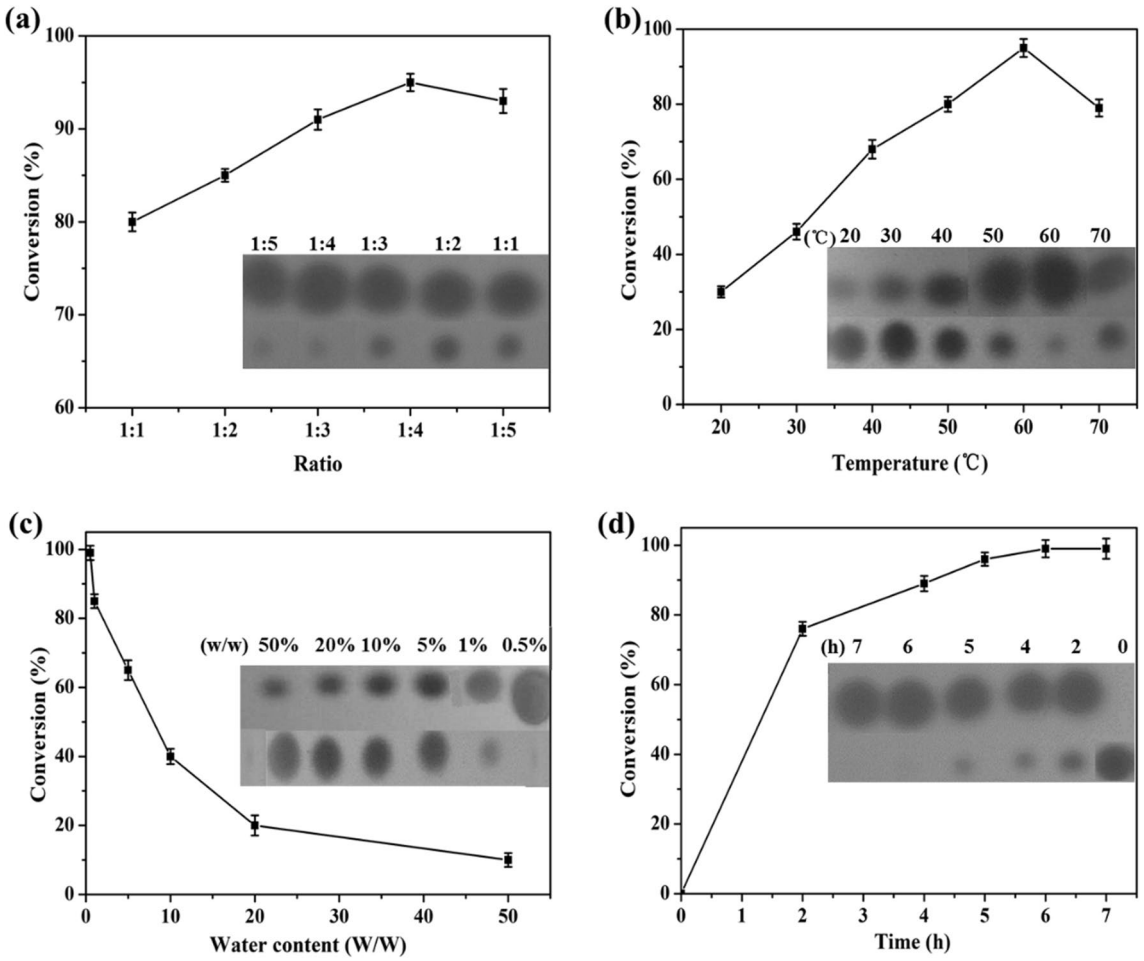


Fig. 6 Optimization of cinnamyl acetate synthesis by immobilized Est_{GUZ753} in *n*-hexane system via TLC and HPLC. **a** Effect of molar ratio on conversion of cinnamyl alcohol with immobilized Est_{GUZ753}; **b** Effect of temperature on conversion of cinnamyl alcohol; **c** Effect of water content on conversion of cinnamyl alcohol with immobilized Est_{GUZ753}; **d** Effect of time on conversion of cinnamyl alcohol with immobilized Est_{GUZ753}. In the TLC, the lower dots represented the substrate content, and the upper dots represented the product content

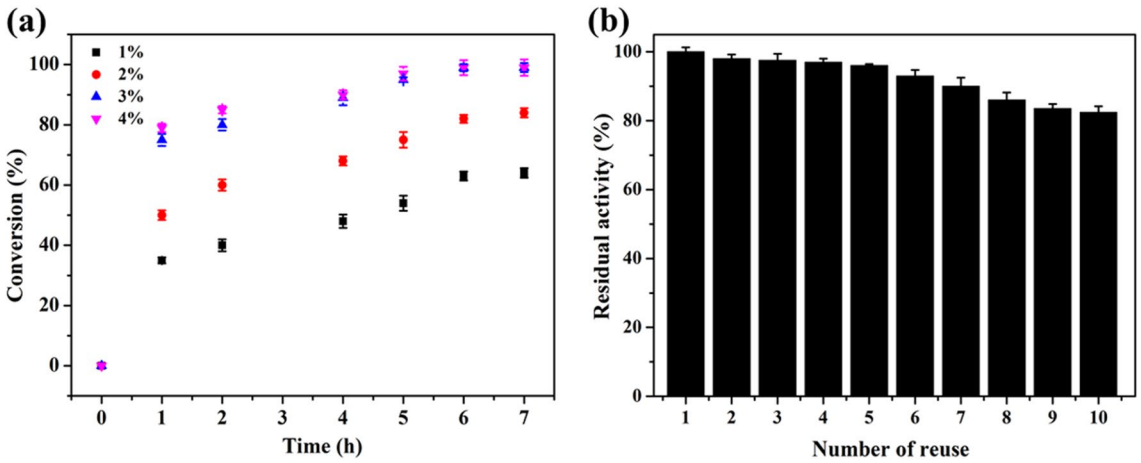


Fig. 7 Optimization of biocatalyst dosage and reusability of the immobilized Est_{GUZ753}. **a** Time course of cinnamyl acetate synthesis with different amounts of immobilized Est_{GUZ753}. Reaction conditions: vinyl acetate as acyl donor; *n*-hexane as solvent; molar ratio, 1:4; temperature, 60 °C; **b** Reusability of the immobilized Est_{GUZ753}

with some previous studies that synthetic activity of most lipases was optimal at relatively low water content in organic systems (typically below 1% (w/w)) [38]. This was due to a larger amount of water in organic medium would reduce the solubility of the substrates and products. Therefore, the synthesis of cinnamyl acetate should be carried out in a micro-water system [39–41].

The reaction time was another key factor for the conversion. The effect of reaction time was analyzed under optimal conditions (*n*-hexane as solvent, molar ratio = 1:4, temperature = 60 °C). As shown in Fig. 6d, the conversion presented an upward trend from 0 to 6 h, and a conversion of 99% was obtained at 6 h. So 6 h was the optimal time. In addition, a comparison of conversion at different concentrations of cinnamyl alcohols was shown in Table S3. The comparison results showed that immobilized Est_{GUZ753} could make about tenfold concentrations of cinnamyl alcohol fully converted to cinnamyl acetate in only 6 h at 60 °C. The result revealed that immobilized Est_{GUZ753} had a high catalytic efficiency at higher temperature.

In the case of industrial production, the increase of enzyme dosage will accelerate the reaction rate and enhance the conversion, but the enzyme dosage determined the cost of the production process. Figure 7a showed that the conversion of cinnamyl alcohol increased with the enzyme dosage from 1 to 3%, and 99% conversion was obtained when the enzyme dosage was 3%. Increasing the enzyme dosage up to 4%, the reaction rate was accelerated at the initial time, but the maximum conversion (99%) almost as the same time as that of 3% enzyme dosage reached. This phenomenon was due to excessive enzymes would aggregate together, which resulted in limited exposed surface area of catalyst with reactants and accordingly decreased mass transfer [42].

The reusability of the catalyst was another important factor in industrial applications. As can be seen in Fig. 7b, immobilized Est_{GUZ753} had a good reusability, which could retain a high activity (80%) after 10 recycles. The decline in activity could be due to partial enzyme leakage from the carrier. After the reaction, the catalyst can be separated and reused in subsequent experiments [43].

4 Conclusions

In this research, the novel esterase gene est_{GUZ753} from *G. uzenensis* DSMZ 13551 had a highly expression in *P. pastoris* KM71 and retained a high activity. Est_{GUZ753} exhibited an excellent thermostability. The $t_{1/2}$ at 70 °C was 28 h. Est_{GUZ753} also had good tolerance towards methanol compared to other thermophilic esterase. The $t_{1/2}$ of Est_{GUZ753} in 90% methanol was 20,160 min. Compared with free Est_{GUZ753}, the immobilized Est_{GUZ753} showed a wider temperature, pH rage and better thermostability. Furthermore, the immobilized Est_{GUZ753} was an efficient catalyst for cinnamyl ester synthesis. A conversion of 99% was achieved in 6 h, and the high concentration of cinnamyl alcohol (1 M) is reported for the first time. The immobilized Est_{GUZ753} also showed a good reusability. On the basis of the excellent characteristics of Est_{GUZ753} reported in this study, Est_{GUZ753} will be a promising catalyst for other organic synthesis.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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