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ORIGINAL PAPER

Enzymatic Synthesis of Feruloylated Lipids: Comparison of the Efficiency of Vinyl Ferulate and Ethyl Ferulate as Substrates

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Abstract A facile and efficient enzymatic synthesis approach to synthesize feruloylated lipids, which are composed of 1(3)-feruloyl-monooleyl-glycerol and 1(3)-feruloyl-dioleyl-glycerol, through lipase-catalyzed transesterification using vinyl ferulate (VF) and ethyl ferulate (EF) as substrate, respectively, with triolein was developed. When VF was used as substrate, a maximum of conversion yield of 91.1% was obtained at 55 °C, 20 mg/mL enzyme content, water activity (a_w) = 0.07, 62 h. This was greater than that when EF was used as substrate (69.6%, 50 °C, 33.3 mg/mL enzyme content, a_w = 0.07, 96 h). *Candida antarctica* lipase (Novozym 435) can be reused for 13 runs without evident loss in activity and stability when VF was used as substrate. The results demonstrate that VF has greater synthetic efficiency and it provides another effective approach to prepare feruloylated lipids under normal pressure, making industry application feasible.

Keywords Ethyl ferulate · Feruloylated lipids · Lipase-catalyzed · Transesterification · Vinyl ferulate

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Introduction

Ferulic acid (4-hydroxy-3-methoxy cinnamic acid, FA), is a phenolic compound that is abundant in plant cell walls. It has been shown to have many physiological functions, including antioxidant, antimicrobial, anti-inflammatory, anti-allergic, antiviral, anti-carcinogenic, free radical scavenging, and UV filter properties, which has made FA widely used in the food, cosmetics, and pharmaceutical industries [1–5]. However, a major obstacle in the application of FA in oil-based food processing and other corresponding industries is its low solubility and stability in hydrophobic media.

To overcome this limitation the modification of FA through esterification with aliphatic alcohols or transesterification with triacylglycerols has been widely reported [6–9]. Chemical synthesis of feruloylated lipids is difficult due to the heat-sensitivity and susceptibility of FA to oxidation at high temperatures and under certain pH conditions [10–12]. The advantages associated with enzymatic synthesis include mild-operating reaction conditions, high specificity, and easy recovery of the end-product [13]. For this reason, many groups have concentrated their attention on the enzymatic synthesis of feruloylated lipids [1, 2, 10, 13–19] and all substrates used are either FA or ethyl ferulate (EF). Sun et al. [20] previously reported an enzymatic route for synthesis of feruloylated lipids, which consists of two steps, namely, transesterification of EF and glycerol, followed by esterification of oleic acid with 1-glyceryl ferulate. Although a feruloylated lipids yield as high as 96% is obtained under reduced pressure condition, excessive quantities of non-reacted glycerol have a detrimental influence upon the activity of lipase [19]. Furthermore, from an economic point of view, even though the yield is relatively high, it is

uneconomic to introduce the additional step and the use of reduced pressure on the grounds that more energy is required and it is hard to apply this strategy to industry applications.

In this work, we aimed to enhance the yield of feruloylated lipids with one-step reaction under atmospheric pressure in order to make industrial application possible. To reach this target, we have altered the substrate EF previously used by all of the investigated synthetic routes to vinyl ferulate (VF), which has not been used for the synthesis of feruloylated lipids, through lipase-catalyzed transesterification. The feruloylated lipids consisted of 1(3)-feruloyl-monooleyl-glycerol (FMOG) and 1(3)-feruloyl-dioleyl-glycerol (FDOG). One of the advantages of VF is that the vinyl alcohol released from the transesterification reaction tautomerizes to acetaldehyde which makes the process irreversible [21] and this improvement benefits the yield of feruloylated lipids and the reaction time. The optimal reaction conditions were obtained by investigation of reaction factors, including reaction time, temperature, water activity, enzyme content and enzyme stability. The high synthetic efficiency of VF as substrate has been further confirmed by comparative studies when EF was used as the substrate under the same conditions. The two synthesis schemes of feruloylated lipids are shown in Fig. 1 and the synthesis scheme of VF is shown in Fig. 2.

Materials and Methods

Enzyme and Chemicals

Novozym 435 (*Candida antarctica* lipase immobilized on polyacrylic resin with an activity of 10,000 propyl laurate units, PLU/g solid enzyme) was obtained from Novozymes A/S (Bagsvaerd, Denmark). FA (purity >99%) and EF (purity >99%) were from the Suzhou Chang Tong Chemical Co., Ltd. (Suzhou, China). Triolein (TO) (purity >99%) was obtained from the Sigma Corporation (USA). VA (purity >99%) was from the Shanghai Qinxi Chemical Technology Co., Ltd. (Shanghai, China). Silica gel (200–300 mesh) was provided by the China National Medicines Corporation Ltd. (Shanghai, China).

Methanol and glacial acetic acid were of HPLC grade and all other solvents were of analytical grade and were dried by activated 4-Å molecular sieves before use. All the enzymes were used directly in commercial preparation without further purification. Other reagents used were obtained from commercial suppliers and were of reagent grade unless otherwise stated.

Preparation, Purification and Identification of Vinyl Ferulate

Concentrated sulfuric acid (0.2 mL) was added dropwise to 3.98 g (0.02 mol) FA and 0.22 g $Hg(OAc)_2$ in 68 mL VA.

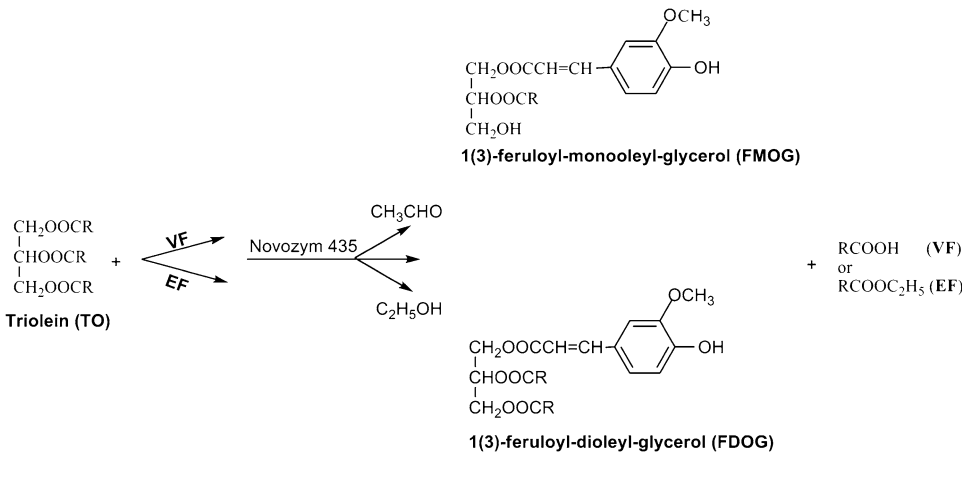

Fig. 1 Two schemes for the preparation of feruloylated lipids

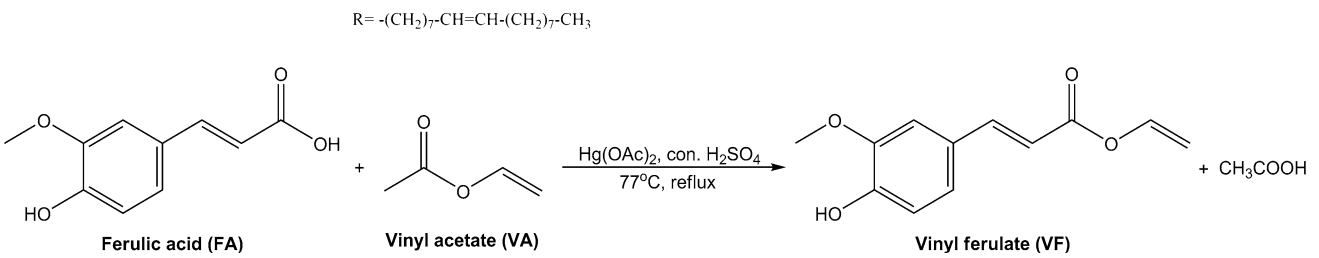

Fig. 2 Scheme for the preparation of vinyl ferulate

The mixture was stirred at room temperature for 30 min and then heated under reflux (77 °C) for 3 h (Fig. 2). After cooling to room temperature, 2.0 g anhydrous Na_2SO_4 was added. VF was obtained after purification by column chromatography [silica gel (200–300 mesh), petroleum ether/ethyl acetate (9:1, v/v) as the mobile phase].

The structure of vinyl ferulate was determined by ^{1}H NMR and ^{13}C NMR (Bruker DRX 400 MHz NMR spectrometer, Germany) at 400 and 100.5 MHz, respectively. $CDCl_3$ was used as the solvent. Chemical shifts are given in ppm and relative to TMS as internal standards. The electrospray ionization-mass spectra (ESI–MS) were recorded on a Shimadzu LCMS-QP 2010 spectrometer.

^{1}H NMR ($CDCl_3$, δ, ppm): 7.72 (t, 1H, –OCH=), 7.41 (d, 1H, Ar–C<u>H</u>=), 7.04 (m, 3H, Ar–H), 6.37 (d, 1H, =CH–CO), 4.89 (m, 2H, $=CH_2$), 3.87 (s, 3H, $–OCH_3$). ^{13}C NMR ($CDCl_3$, δ, ppm): 168.2 (–CO–), 145.0 (Ar–<u>C</u>H=), 150.1, 146.5, 128.9, 121.3, 115.8, 110.3 (Ar), 140.5 (–O–<u>C</u>H=CH_2), 114.5 (Ar–CH=<u>C</u>H–), 96.7 ($=CH_2$), 54.9 ($–OCH_3$).

ESI–MS (m/z): 208 $[M + H]^+$.

General Procedure for the Enzymatic Synthesis and Batch Reaction Operation

According to our previous study [1], the yields of feruloylated lipids are the best when the water activity (a_w) is 0.23, so we chose it as the initial condition when the influence of water activity is unknown in this study. Conditions for the transesterification of both VF and EF with TO were as follows: 0.25 mmol VF or EF was reacted with 0.75 mmol TO in 3 mL toluene, followed by various contents of Novozym 435 (3.3–40 mg/mL) at a temperature range from 40 to 55 °C, a_w value from 0 to 0.97, 210 rpm.

In the batch reaction, the lipase particles were filtered off with toluene three times after the completion of the reaction and then added into fresh reaction medium directly to catalyze a second run.

Purification of Feruloylated Lipids

When the transesterification reaction was complete, the enzyme was removed by filtration and the filtrate was concentrated under a vacuum. The residue was separated and purified by flash column chromatography (silica gel 200–300 mesh) using dichloromethane/benzene/ether/hexane (5:3:2:1, v/v/v/v) as the mobile phase to obtain FDOG and then the mobile phase was changed to ethyl acetate/hexane (4:1, v/v) to isolate FMOG.

HPLC Analysis

HPLC analysis was performed using a Waters 510 with an Inertsil Ph-3 column (4.6 mm ID × 250 mm, 5 μm, GL Sciences, Japan) fitted with a dual absorbance detector (Waters 2487) at 325 nm. Solvents were filtered (Whatman 0.45 μm nylon membrane filters) and degassed using a Thermo Separation Products SCM 1000 Membrane Degasser before use. The mobile phases were solvent A (water containing 0.1% glacial acetic acid) and solvent B (100% methanol), operating at 0.5 mL/min flow as a linear gradient [100% (v/v) B to 50% (v/v) B] for 10 min, then to 100% (v/v) B for a further 15 min. This was followed by isocratic flow of 100% (v/v) B for 35 min.

Sample (10 μL) was removed from the reaction mixture at the set times during the transesterification process and further diluted 100-fold with methanol and then passed through organic-phase syringe filter (13 mm × 0.45 μm, Nylon, Shanghai ANPEL, China) prior to injection. The pretreated sample injected was 10 μL.

According to our previous study [1] the conversion yields of two reactions were determined by measuring residual VF or EF and FA peak area by HPLC and the yields of FMOG and FDOG of two reactions were calculated as the percentage ratio of peak areas of the FMOG and FDOG to the peak area of residual VF or EF and FA.

Control of Water Activity

Toluene was dried by gently shaking with 4-Å molecular sieves overnight before use. The initial water activities (a_w) of the substrates, toluene and the enzyme were controlled by gaseous equilibrium with different saturated salt solutions or the solid in separate sealed containers for at least 7 days at 25 °C. The following salts and solid reagent were used: Silica gel ($a_w = 0$), LiBr ($a_w = 0.07$), LiCl ($a_w = 0.11$), CH_3COOK ($a_w = 0.23$), $Mg(NO_3)_2$ ($a_w = 0.53$), NaCl ($a_w = 0.75$), $K_2Cr_2O_7$ ($a_w = 0.97$).

Results and Discussion

Investigation of Reaction Time

Figure 3 shows the reaction progress versus reaction time of two reactions. There was a distinct increase in the conversion yield in VF up to 62 h and then it remained relatively constant (Fig. 3a). By contrast, the conversion yield using EF increased up to 96 h and then remained constant. The differences for VF and EF in reaching reaction equilibrium could be attributed to the acetaldehyde produced in VF not participating in the reaction while the alcohol formed in EF shifts the reaction equilibrium towards a reverse reaction.

The yields of FMOG and FDOG obtained from the two reactions are given in Fig. 3b. In VF, the yield of FMOG showed a slight increase over the time course, from 30.4%

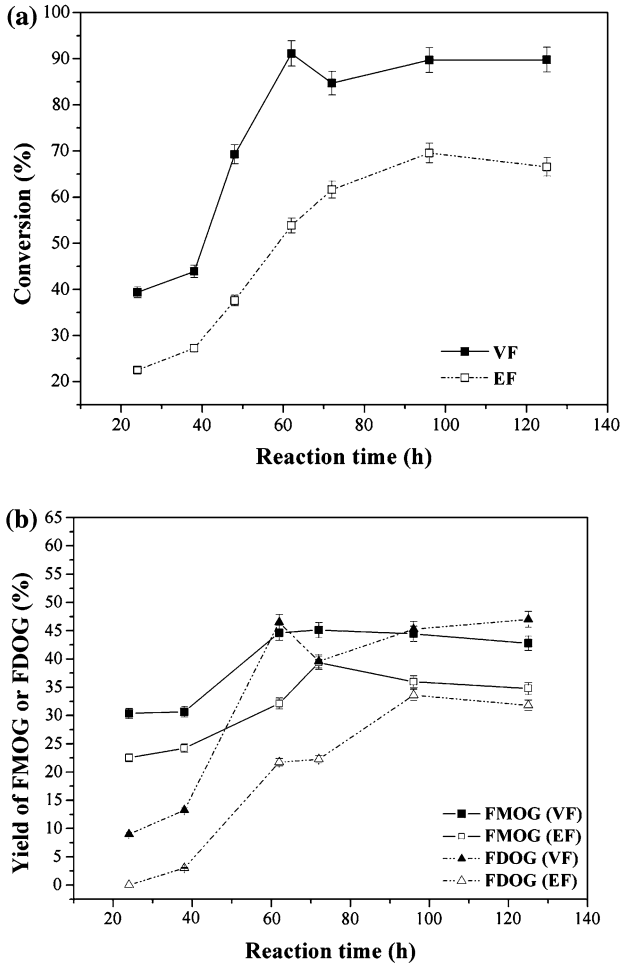

Fig. 3 Investigation of time course on lipase-catalyzed feruloylated lipids of two reactions. **a** Conversion yields of two reactions (*filled squares*) VF, (*open squares*) EF. **b** Yields of FMOG and FDOG of two reactions (*filled squares*) FMOG of VF, (*open squares*) FMOG of EF, (*filled triangles*) FDOG of VF, (*open triangles*) FDOG of EF (reaction conditions: toluene 3 mL, VF or EF 0.25 mmol, TO 0.75 mmol, 55 °C, Novozym 435 40 mg/mL, $a_w = 0.23$, 210 rpm). Experiments were carried out in triplicate

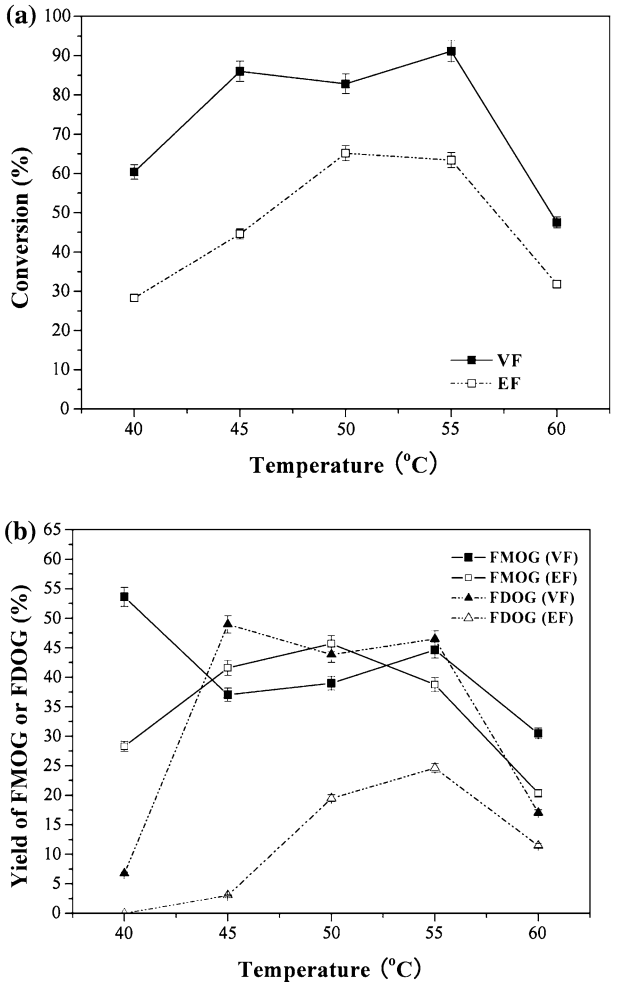

Fig. 4 Investigation of reaction temperature on yield of lipase-catalyzed feruloylated lipids of two reactions. **a** Conversion yields of two reactions (*filled squares*) VF, (*open squares*) EF. **b** Yields of FMOG and FDOG in two reactions at the maximum of reaction (*filled squares*) FMOG of VF, (*open squares*) FMOG of EF, (*filled triangles*) FDOG of VF, (*open triangles*) FDOG of EF (reaction conditions: toluene 3 mL, VF or EF 0.25 mmol, TO 0.75 mmol, Novozym 435 40 mg/mL, $a_w = 0.23$, 210 rpm, 62 h). Experiments were carried out in triplicate

(min) to 45.1% (max), and then decreased slightly. Similarly, the yield of FMOG from EF demonstrated the same trend, from 22.5% (min) to 39.3% (max). For the overall process the yield of FMOG in VF was always higher than the other procedure (EF).

The yield of FDOG in VF increased significantly up to 62 h to give a maximum yield of 47.0% and then it remained constant. However, for EF, the maximum yield was only 33.6% and this was achieved over a longer time period (96 h).

Investigation of Reaction Temperature

Temperature influences lipase-catalyzed reactions due to the heat-sensitivity of the enzyme. Figure 4 depicts the trend of the conversion yields of two reactions, yields of FMOG and FDOG over a temperature range from 40 to 60 °C. From Fig. 4a, it can be noted that over this temperature range the conversion yield for EF increased with an increase in the reaction temperature up to 55 °C. Whereas the conversion yield for VF increased with increasing temperature below 45 °C and then remained constant until reaching a maximum yield (91.1%) at 55 °C. At the same temperature the conversion yield from VF was greater. It can be seen that increasing the temperature has a positive effect on the yield. This may be due to the increased activity of lipase before the optimum temperature of the enzyme when the temperature rises. However, a higher temperature (60 °C) caused a significant drop in both reaction conversions. This may be explained by partial lipase deactivation at higher temperatures.

The maximum yields of FMOG and FDOG formed by the two reactions are shown in Fig. 4b. Results show that yields of FMOG in VF fluctuate over the temperature range from 40 °C to 60 °C. To be specific, the yield decreased from the its maximum yield of 53.6% at 40 °C to 37.0% at 5 °C and increased slightly to 44.6% at 55 °C. After that, the yield dropped to its minimum value of 30.5% at 60 °C. On the other hand, the yield of FMOG in EF increased with increasing of the temperature until it reached its maximum yield of 45.7% at 50 °C and then it decreased to its minimum value of 20.4% at 60 °C.

As for the yield of FDOG, it can be seen from Fig. 4b that the yield of FDOG in VF increased with increasing temperature and then remained relatively constant after 45 °C. In contrast, the yield of FDOG in EF increased with increasing temperature and reached its maximum yield at 55 °C. Both VF and EF showed a great decrease after 55 °C. It is known from these results that VF reaches its reaction equilibrium sooner in the conversion of FDOG. It is interesting that at each temperature tested the yield of FDOG in VF was greater.

Investigation of Water Activity

Water activity (a_w) is a dimensionless quantity used to represent the energy status of the water in a system. It has been proved that water activity is an important consideration for a biocatalyst in a nonaqueous medium [1, 22–25].

The lipase-catalyzed transesterification of VF and EF with TO at seven different a_w values in toluene was studied (Fig. 5). Results from Fig. 5 indicate very low enzyme activity without water ($a_w = 0$) for both the reactions, which supports the fact that a minimum amount of water is required to activate the enzyme. With the increased addition of water there was a significant increase in yield for both the reactions. When a_w continuously increased, the conversion yields for both reactions decreased, from 96.7 to 42.6% and from 79.8 to 30.9% for VF and EF, respectively. This may be due to excess water being present in the enzyme catalyst particles preventing the transfer of substrates and as the size of the water layer of enzyme increases it is harder for the substrate to reach the catalyst [23]. Furthermore, it is worth noting that the conversion yield from VF is higher at any given a_w value. This suggests that water activity is more influential in EF.

Figure 5b shows the yields of FMOG and FDOG in the two reactions under different a_w values. As for FMOG, the maximum yields were as high as 46.6% at $a_w = 0.11$ and 50.9% at $a_w = 0.07$ were obtained for VF and EF, respectively. FDOG (Fig. 5b) shows that the product yields, irrespective of the methods used, increased as the a_w value increased from 0 to 0.07, then was followed by a decrease from 59.4% (max) at $a_w = 0.07$ to 36.3% at

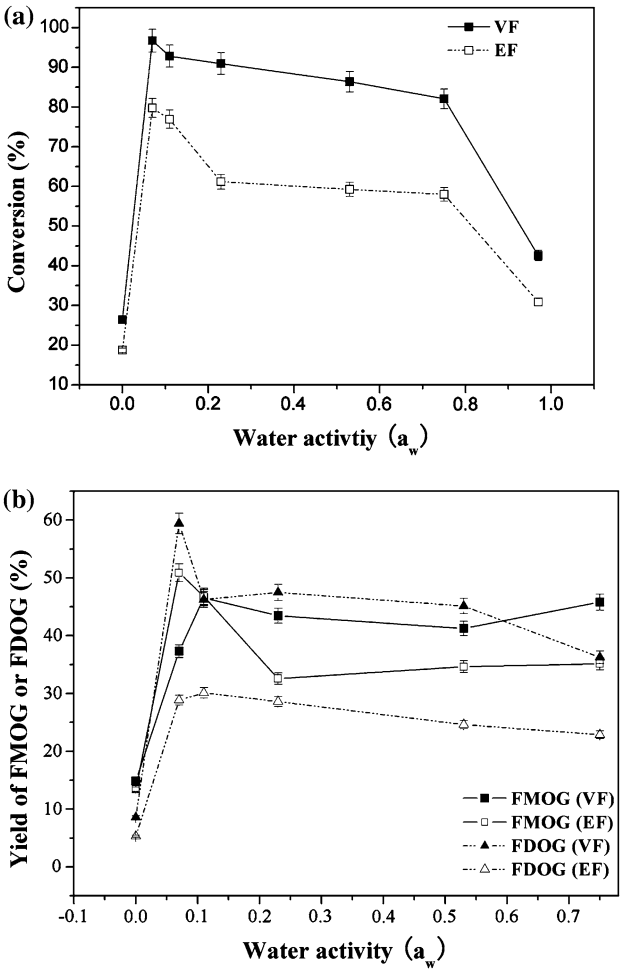

Fig. 5 Investigation of water activity on yield of lipase-catalyzed feruloylated lipids of two reactions. **a** Conversion yields of two reactions (*filled squares*) VF, (*open squares*) EF. **b** Yields of FMOG and FDOG in two reactions (*filled squares*) FMOG of VF, (*open squares*) FMOG of EF, (*filled triangles*) FDOG of VF, (*open triangles*) FDOG of EF (reaction conditions: toluene 3 mL, VF or EF 0.25 mmol, TO 0.75 mmol, 55 °C Novozym 435 40 mg/mL, 210 rpm, 62 h). Experiments were carried out in triplicate

$a_w = 0.75$ and from 30.1% (max) at $a_w = 0.11$ to 22.9% at $a_w = 0.75$ for VF and EF, respectively. It is worth noting that the yield of FDOG from VF is higher than EF at any of the a_w values tested. This may be also ascribed to the effects of different by-products that are produced by the two reactions.

Investigation of Enzyme Content

The amounts of the enzyme required for the reactions of two reactions are shown in Fig. 6. Conversion yields as high as 90.2% at 20 mg/mL enzyme and 61.9% at 33.3 mg/mL enzyme were observed for VF and EF, respectively. For VF, the conversion yield decreased when the enzyme content exceeded 20 mg/mL, while the conversion yield in

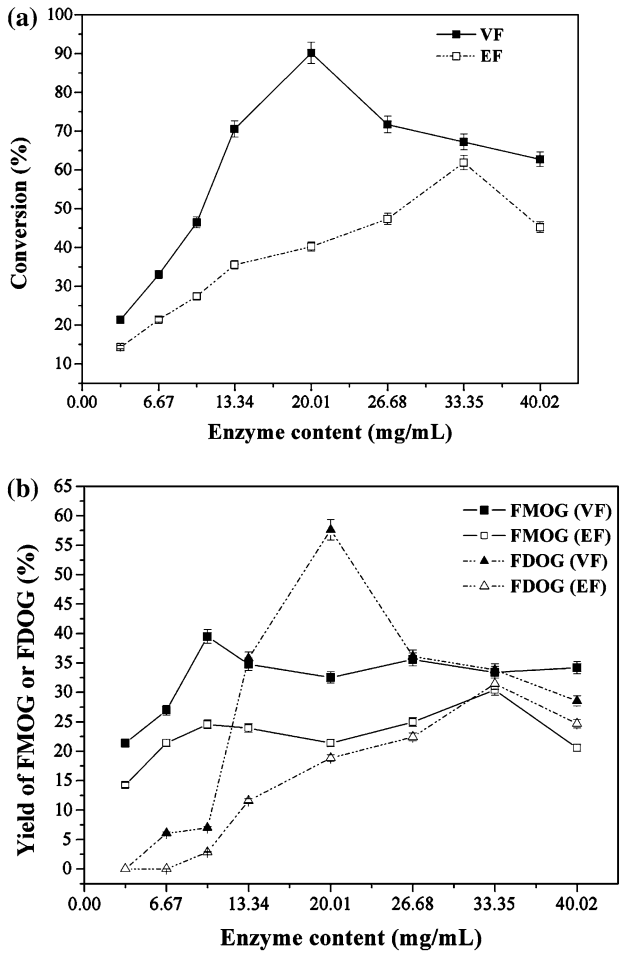

Fig. 6 Investigation of enzyme content on yield of lipase-catalyzed feruloylated lipids of two reactions. **a** Conversion yields of two reactions (*filled squares*) VF, (*open squares*) EF. **b** Yields of FMOG and FDOG of two reactions at the maximum rate of reaction (*filled squares*) FMOG of VF, (*open squares*) FMOG of EF, (*filled triangles*) FDOG of VF, (*open triangles*) FDOG of EF (reaction conditions: toluene 3 mL, VF or EF 0.25 mmol, TO 0.75 mmol, 55 °C, $a_w = 0.07$, 210 rpm, 62 h). Experiments were carried out in triplicate

EF decreased when the enzyme content was above 33.3 mg/mL. Remarkably, the higher content of enzyme lowered the product yield and this was presumably due to increasing viscosity effects that can reduce the reaction rate. Similar observations have also been reported by Shaw [26].

Figure 6b displays the yields of FMOG and FDOG of two reactions. In VF the maximum yield (39.5%) of FMOG was observed at 10 mg/mL enzyme content, however, in EF the highest yield (30.4%) of FMOG was at 33.3 mg/mL enzyme. For FDOG, the maximum yield (57.6%) from VF was observed at 20 mg/mL enzyme and then it decreased while the maximum yields (31.5%) from EF was noted at 33.3 mg/mL enzyme and then it decreased. Moreover, Fig. 6b also demonstrates that at any given enzyme content

Table 1 The yields of FMOG and FDOG in Reaction A after each operation run

Run	The yield of product (%) ± SD		
	Total yield	Yield of FMOG	Yield of FDOG
1	89.2 ± 2.7	36.2 ± 1.1	53.4 ± 1.6
2	94.0 ± 2.8	36.3 ± 1.1	57.8 ± 1.7
3	90.6 ± 2.7	36.8 ± 1.1	53.9 ± 1.6
4	89.1 ± 2.7	34.0 ± 1.0	55.1 ± 1.7
5	88.4 ± 2.7	35.0 ± 1.1	53.4 ± 1.6
6	83.5 ± 2.5	35.1 ± 1.1	48.3 ± 1.5
7	84.3 ± 2.5	35.1 ± 1.1	49.1 ± 1.5
8	77.4 ± 2.3	35.6 ± 1.1	41.8 ± 1.3
9	78.3 ± 2.4	33.0 ± 1.0	45.2 ± 1.4
10	75.8 ± 2.3	32.8 ± 1.0	43.0 ± 1.3
11	75.0 ± 2.3	33.2 ± 1.0	41.7 ± 1.3
12	78.6 ± 2.4	34.5 ± 1.0	44.1 ± 1.3
13	77.6 ± 2.3	33.8 ± 1.0	43.8 ± 1.3

Reaction conditions: toluene 3 mL, VF 0.25 mmol, TO 0.75 mmol, 55 °C, Novozym 435 20 mg/mL, $a_w = 0.07$, 210 rpm, 62 h

SD standard deviation

value, the yield of FMOG from VF was always greater than that from EF, which is consistent with results provided above.

Investigation of Enzyme Stability

It is essential to investigate the reusability of enzymes because of their high cost and instability under operational conditions in the industrial applications. Experiments were carried out in triplicate. Results showed that there was no significant loss of lipase activity when the lipase was reused 13 times (Table 1). The total conversion yield still remained above 75.0%, and the yield of FMOG and FDOG remained above 32.8 and 41.7%, respectively. This excellent stability of Novozym 435 in organic media can be helped by the relatively mild operation conditions used and the solubility of substrates.

Conclusions

In the present study, we described an improved enzymatic synthesis protocol for the preparation of feruloylated lipids through transesterification of VF and TO in toluene catalyzed by Novozym 435. We compared the synthetic efficiency of two reactions, which used VF and EF as substrates adopting the same conditions throughout, including: reaction time, temperature, water activity and enzyme content. In addition, enzyme stability was studied in VF. From our observations it can be concluded that

regardless of the experimental conditions used greater effectiveness and efficiency were achieved when VF was adopted as the substrate in feruloylated lipids synthesis. The outcomes from these experiments could provide a useful platform for developing future strategies of feruloylated lipids preparation.

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