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Enzymatic synthesis and characterization of novel feruloylated lipids in selected organic media

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ABSTRACT

A facile enzymatic synthesis approach to prepare novel feruloylated lipids through the lipase-catalyzed transesterification reaction of ethyl ferulate (EF) with tributyrin (TB) in toluene was investigated. The nuclear magnetic resonance (NMR) and electrospray ionization-mass spectroscopy (ESI-MS) analysis confirmed the formation of two major products, 1(3)-feruloyl-monobutyryl-glycerol (FMB) and 1(3)-feruloyl-dibutyryl-glycerol (FDB). The influences of different enzyme source, organic solvent, molar ratio, reaction temperature, agitation speed and water activity on the total conversion of reaction, distribution of FMB and FDB and selectivity of these two novel derivatives of FA were analyzed systematically. Under the optimal conditions, the highest conversion of feruloylated lipids achieved was 73.6%, which was composed of FMB 58.3% and FDB 13.1%, respectively. The enzyme can be reused for 14 runs without evident loss in activity and stability.

1. Introduction

Ferulic acid (4-hydroxy-3-methoxy cinnamic acid, FA), an effective component of Chinese medicine herbs such as *Angelica sinensis* and *Cimicifuga heracleifolia*, is a phenolic acid. It has shown a strong antioxidant capacity and other physiological activities, including anticarcinogenic [1], anti-inflammatory [2], and anti-Alzheimer's diseases [3]. Besides, it can be used as a potential UV-absorbing ingredient in cosmetic due to its absorbance of UVA and UVB [4,5]. However, FA exhibits low solubility and stability in hydrophobic media, which in turn reduces its biological efficiency in lipophilic media such as oils, emulsions and fats. To overcome this limit, modification of FA via its esterification with aliphatic alcohol has been widely investigated [6–10]. Also some studies on modifying long-chain triglycerides with FA have been reported [11–15]. Nevertheless, to our knowledge, there are few reports on the modification of short-chain triglyceride to produce feruloylated lipids.

Structuring tributyrin (TB) with FA could potentially result in novel feruloylated lipids. TB is a healthy short-chain triglyceride as a liquid fat existing in spice plants. The relatively short acyl group length allows low viscosity, low melting point and low calorie compared to long-chain triglyceride. On the other hand, TB is a pro-drug of butyric acid from a nutritional and pharmacological point of view. It has been well reported bearing anti-tumor properties and inhibiting the proliferation [16]. Thus, we reasoned that structuring TB with FA could produce novel bifunctional feruloylated lipids, because FA moiety works as a natural antioxidant, while the butyric moiety is a traditional anti-tumor agent (Scheme 1). In addition, the relationship between distributions of FA on the backbone of TB and their different solubility is a valuable and interesting topic, which would provide available information to further investigate the pharmacological benefits of these novel feruloylated derivatives.

Chemical synthesis of feruloylated lipids is limited due to the heat sensitivity and oxidation susceptibility of FA. In addition, using high temperature frequently causes a dark color, burnt taste, and high energy consumption [17]. In contrast, lipase-catalyzed reaction has been of great interest because of the mild operating conditions, the high substrate specificity and positional selectivity. On the other hand, enzymatic synthesis in organic media may permit an increase in the solubility of both hydrophobic and hydrophilic substrates and an improvement in enzyme stability with the additional advantage of simple recovery of reaction products.

In this study, selected nutritional and functional feruloylated lipids, which was composed of 1(3)-feruloyl-monobutyryl-glycerol (FMB) and 1(3)-feruloyl-dibutyryl-glycerol (FDB) was synthesized by a facile and efficient enzymatic synthesis method. The effects of reaction parameters, including source of enzyme, organic solvent, substrate molar ratio, reaction temperature, agitation speed and water activity on the conversion and distribution of FDB and FMB

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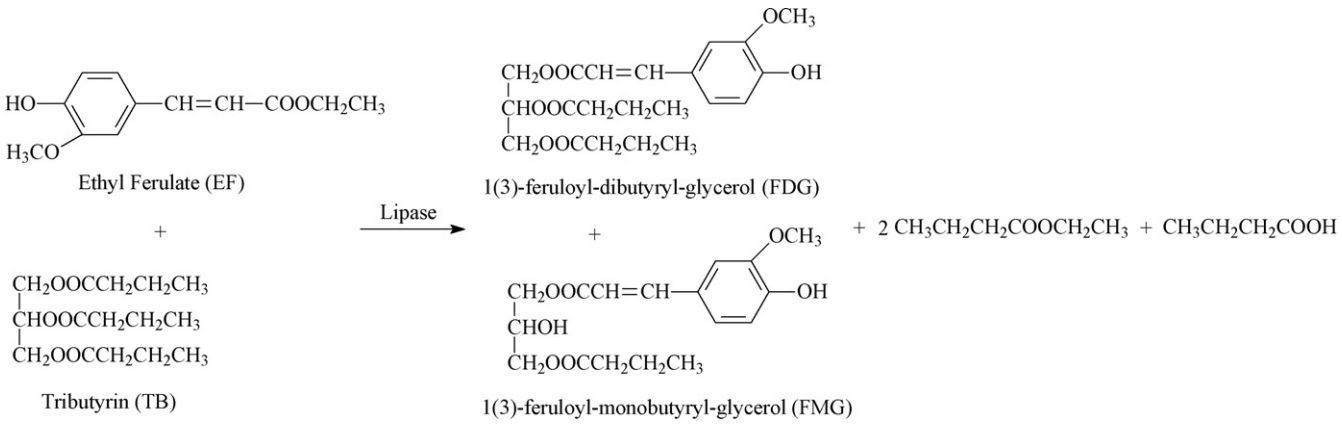

Scheme 1. Reaction scheme of lipase-catalyzed transesterification of ethyl ferulate with tributyrin.

were investigated. In addition, the operational life of Novozym 435 was assayed to evaluate the practicality of the developed method.

2. Materials and methods

2.1. Enzymes 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 purchased from Novozymes A/S (Bagsvaerd, Denmark). Lipase from porcine pancreas Type II (EC 3.1.1.3, powder, 30–90 U/mg) and lipase from *Candida rugosa* Type VII (EC 3.1.1.3, powder, 706 U/mg) were purchased from Sigma. Lipase from *Pseudomonas fluorescens* (EC 3.1.1.3, powder, 2.2 U/mg), lipase from *Candida cylindracea* (EC 3.1.1.3, powder, 5.18 U/mg), lipase from *Mucor javanicus* (EC 3.1.1.3, powder, 8.6 U/mg), lipase from *Aspergillus niger* (EC 3.1.1.3, powder, 187 U/g) and lipase from *Rhizopus oryzae* (EC 3.1.1.3, powder, 55.7 U/mg) were purchased from Fluka Chemical Co. Lipase from *Pseudomonas cepacia* (Amano "PS") were purchased from Amano International Enzyme Co. (Nagoya, Japan). FA (purity > 99%) and ethyl ferulate (EF) (purity > 99%) were purchased from Suzhou Chang Tong Chemical Co., Ltd. (Suzhou, China). TB (purity > 99%) was purchased from Tao He Chemical Co., Ltd. (Shanghai, China).

All the 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. All other reagents used were obtained from commercial suppliers and were of reagent grade unless otherwise noted.

2.2. Control of the initial water activity

The solvents were dried by gentle shaking with 4 Å molecular sieves overnight. The initial water activities (a_w) of the anhydrous solvents, the substrates and the enzymes were controlled by gaseous equilibrium with different saturated salt solutions in separate closed containers for 120 h at 25 °C. The following salts were used: LiBr (a_w = 0.07), LiCl (a_w = 0.11), CH_3COOK (a_w = 0.23), $MgCl_2$ (a_w = 0.33), K_2CO_3 (a_w = 0.43), $Mg(NO_3)_2$ (a_w = 0.53), NaCl (a_w = 0.75).

2.3. Lipase-catalyzed transesterification

Enzymatic reaction was carried out in a closed, screw-capped tube containing EF and TB, 3 mL organic solvent and 120 mg lipase. The reaction mixture was stirred with a magnetic stirrer at various temperatures. Samples were withdrawn at specified time intervals for measurement of the total conversion and selectivity. Control experiments, without enzyme, were carried out in tandem with reactions using identical conditions. All the experiments were carried out in triplicate.

2.4. Purification and identification of the feruloylated lipids

Upon the completion of the reaction, the enzyme was filtered off, and the filtrate was concentrated under vacuum. The residue was separated and purified through flash column chromatography using benzene/ether/dichloromethane/hexane (3:5:2:2, v/v/v/v) as the mobile phase. The structures of feruloylated lipids were 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 solvent. Chemical shifts were given in ppm relative to TMS as internal standard. The electrospray ionization-mass spectra (ESI-MS) were recorded on a Shimadzu LCMS-QP 2010 spectrometer.

2.4.1. Identification of FDB

^{1}H NMR ($CDCl_3$, δ, ppm): 7.61 (d, 1H, Ar–H), 7.01–7.30 (m, 2H, Ar–H), 6.90 (d, 1H, =C<u>H</u>–Ar), 6.27 (t, 1H, =CH(CO)), 5.37 (m, 1H, –C<u>H</u>$(CH_2O)_2$), 4.32–4.39 (m, 4H, 2-CH_2O), 3.91 (S, 3H, –OCH_3), 2.31–2.35 (m, 4H, 2-C<u>H</u>$_2$$(CH_2CH_3)$), 1.63–1.69 (m, 4H, 2-C<u>H</u>$_2$$CH_3$) and 0.93–0.97 (m, 6H, 2-CH_3). ^{13} C NMR ($CDCl_3$, δ, ppm): 175.5 (–CH_2O<u>C</u>O–), 175.1 (–CHO<u>C</u>O–), 169.0 (–<u>C</u>OCH=), 148.1 (=CH–), 150.7, 148.1, 129.0, 125.6, 116.7, 111.9 (Ar), 71.4 (–<u>C</u>H$(CH_2O)_2$), 64.6, 64.5 (2-<u>C</u>H_2O), 58.3 (OCH_3), 38.4, 38.2 (–<u>C</u>H_2COOCH–, –<u>C</u>$H_2$$COOCH_2$–), 20.7, 20.6 (–<u>C</u>$H_2$$CH_2$COOCH–, –<u>C</u>$H_2$$CH_2$$COOCH_2$–) and 15.9,15.8 (2 CH_3–).

ESI-MS (m/z): 407.9 $[M+H]^+$.

2.4.2. Identification of FMB

^{1}H NMR ($CDCl_3$, δ, ppm): 7.58 (d, 1H, =C<u>H</u>–Ar), 6.84–7.02 (m, 3H, Ar–H), 6.24 (d, 1H, –CH=), 5.86 (m, 1H, –C<u>H</u>$(CH_2O)_2$), 3.83–4.24 (m, 4H, 2-CH_2O–), 3.86 (s, 3H, –OCH_3), 3.82 (d, 1H, –C<u>H</u>(OH)–), 2.28 (t, 2H, –C<u>H</u>$_2$$(CH_2CH_3)$), 1.58–1.63 (m, 4H, 2-C<u>H</u>$_2$$CH_3$)) and 0.89–0.91 (m, 3H, –CH_3). ^{13} C NMR ($CDCl_3$, δ, ppm): 176.1 (–<u>C</u>OCH_2–), 169.6 (–CO<u>C</u>H=), 148.2(=CH–), 150.6, 149.1, 129.1, 125.7, 116.8, 111.8 (Ar), 117.1 (–CH=), 70.9 (–CHOH–), 67.6, 67.5(–CH_2O–), 58.3(–OCH_3), 38.3 (–<u>C</u>H_2CO–), 20.7 (–CH_2–) and 15.9 (–CH_3).

ESI-MS (m/z): 337.9 $[M+H]^+$.

2.5. TLC analysis

Qualitative analysis of the reaction mixtures was made by TLC on silica gel 60 F 254 plates (Merck, Germany), using a solvent mixture system of benzene/ether/dichloromethane/hexane (3:5:2:2, v/v/v/v). The reaction products were detected under UV light (254 nm).

2.6. HPLC analysis

Analytical HPLC was performed using Waters 510 with an Inertsil Ph-3 column (4.6 mm I.D. × 250 mm, 5 μm, GL Sciences, Japan), with a dual absorbance detector (Waters 2487) at 325 nm. The mobile phase was solution A (water containing 0.1% acetic acid) and solution B (100% methanol), in all cases at 1 mL/min flow of A/B (30/70, v/v). Analysis was carried out at room temperature. Solvents were filtered using Whatman 0.45-μm nylon membrane filters (Sigma–Aldrich) and degassed using a Thermo Separation Products SCM 1000 Membrane Degasser.

10 μL sample was removed from the reaction mixture at set time intervals during the reaction and further diluted 100-fold with methanol. The sample injection volume was 10 μL.

According to previous reports [11], the total conversion of reaction was determined most accurate by measuring residual EF and FA peak area by HPLC. The sum of all ferulate species peak areas remained constant over the time course of the reaction, allowing accurate FDB and FMB yields to be calculated as the percentage ratio of the FDB and FMB peak area to the residual EF and FA peak area.

In addition, the selectivity is defined as

$$\text{Selectivity} = \frac{M_1}{M_2} \times 100\%$$

where M_1 and M_2 are yields for FMB and FDB respectively.

3. Results and discussion

3.1. Screening of lipases

Eight commercially available lipases were screened and evaluated for their ability to catalyze transesterification reactions between EF and TB. Reactions were carried out at 50 °C for 4 days using a 1:3 EF/TB molar ratio and 120 mg of the investigated enzymes in 3 mL toluene. In the absence of enzyme, the yield of conversion was less than 1%. Among the assayed lipases, Novozym 435, which is an immobilized form of lipase B from *C. antarctica*, gave the highest 59.1% of conversion (Table 1). Many research groups have demonstrated that immobilized lipase could increase the contact surface area of lipase with substrates. Moreover, enzyme in immobilized form could exhibit more stable than free ones in organic solvents, therefore keeping enzyme activity [18]. On the other hand, free enzyme in organic solvents often forms conglomerates and is denaturalized, resulting in low catalysis activity. Therefore, Novozym 435 was selected as the biocatalyst in the following experiments.

3.2. Effect of organic media

Reaction media play an important role in maintaining enzyme catalytic activity and stability. It also influences the relative solubility of the substrates. In order to investigate the most suitable organic media for the transesterification, nine different solvents with log *P* ranging from −1.3 to 4 were screened to optimize the reaction conditions for enzymatic transesterification. The results are shown in Table 2.

Table 1
Effect of enzyme on lipase-catalyzed feruloylated lipids.

Enzyme	Conversion (%) ± S.D.[a]
Lipase from *Candida cylindracea*	19.2 ± 0.9
Lipase from porcine pancreas	<5
Lipase type VII from *Candida rugosa*	26.2 ± 1.4
Amano lipase M from *Mucor javanicus*	16.7 ± 1.7
Candida antarctica lipase acrylic resin	59.1 ± 1.1
Lipase from *Rhizopus oryzae*	<5
Lipase from *Pseudomonas cepacia*	<5
Lipase from *Aspergillus niger*	<5

Reaction conditions: toluene 3 mL, EF 0.5 mmol, TB 1.5 mmol, Novozym 435 120 mg, 210 rpm, 50 °C, and 4 days.
[a] Standard deviation of triplicate determinations from different experiments.

Table 2
Effect of organic medium on lipase-catalyzed feruloylated lipids.

Solvent	Log *P*[a]	Conversion[b] (%) ± S.D.
DMSO	−1.3	<1
DMF	−1.0	<1
Acetonitrile	−0.39	<1
Tetrahydrofuran	0.46	<5
2-Methyl-2-butanol	0.79	42.1 ± 1.2
Toluene	2.5	59.1 ± 1.7
Cyclohexane	3	39.9 ± 1.9
Hexane	3.5	34.7 ± 2.2
Octane	4.7	19.7 ± 1.4

Reaction conditions: solvent 3 mL, EF 1 mmol, TB 3 mmol, Novozym 435 120 mg, 210 rpm, 50 °C, and 4 days.
[a] Log *P* is the logarithm of the partition coefficient of a given compound in the octanol–water two-phase system.
[b] Conversion were determined by HPLC analysis.

In our study, the highest conversion 59.1% achieved in toluene (log *P* = 2.5), which was higher than the corresponding reactions conversion that used n-hexane (log *P* = 3.5) or octane (log *P* = 4). This is somewhat inconsistent with general reports that solvents with log *P* > 3 are more suitable for enzymatic synthesis of lipids [19]. However, considering the obvious difference in polarity between EF and TB, the above result is reasonable. EF is easy to dissolve in polar solvents, while TB is hydrophobic and preferred by nonpolar solvents. Therefore, organic solvents with moderate polarity are more suitable for the substrate and product solubility. The conversion was less than 1% when the log *P* value of solvent is less than 0. This is probably due to that, the solvents with high polarity may strip water from enzyme molecules easily, and the enzyme could not get enough water for keeping its active configuration [20]. This could be the reason why almost no product was detected in organic solvent with a very low log *P* value. Consequently, toluene was used as reaction medium in the following assays.

3.3. Effect of reaction time on transesterification

Fig. 1 depicts the time course of the product of total feruloylated lipids, FDB and FMB formation over a 10-day period of lipase-

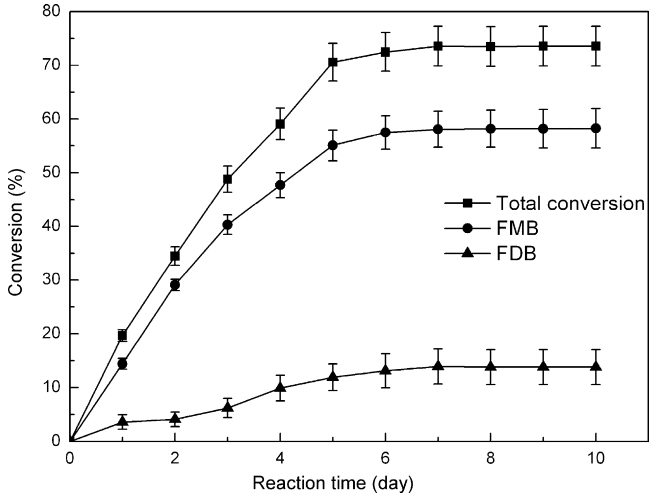

Fig. 1. Time course for lipase-catalyzed feruloylated lipids. (Reaction conditions: toluene 3 mL, EF 0.5 mmol, TB 1.5 mmol, 50 °C, Novozym 435 120 mg, a_w 0.23, 210 rpm.)

catalyzed reaction. The results (Fig. 1) showed that there was a drastic increase in feruloylated lipids after 5 days and remained relatively constant beyond this period. The limited increase in the conversion yield after 5 days may be due to the reaction having reached its equilibrium. The results also indicated that the conversion yields of FDB and FMB showed the same trends over the time course of the reaction, and a higher yield for FMB was observed (58%) compared to 13.8% for FDB after 10 days. Similar phenomenon has also been reported by Sabally et al. for the lipase-catalyzed transesterification of triolein or trilinolenin with selected phenolic acids [13].

3.4. Influence of substrate molar ratio

The effect of molar ratio of EF to TB on the lipase-catalyzed transesterification was investigated (Fig. 2a). When the molar ratio of EF to TB were set at 1:1 and 1:2, the maximum conversion yield achieved 42.5% and 49.2% respectively after 5 days and remained relatively constant beyond this period. The highest conversion yield of 73.6% was obtained with EF to TB ratio of 1:3 after 7 days reaction. In theory, one mole butyric acid is liberated from hydrolysis of TB when one molar ratio of TB to EF reaction (Scheme 1). Thus, the decrease in conversion yield, obtained at ratios of 1:4, 1:6 and 1:8 after 7 days reaction could be due to a shift in the thermodynamic equilibrium reaction, which may be promoted by an increase in the concentrations of butyric acid resulted from the hydrolysis of TB. The accumulation of butyric acid could produce a hydrophilic hindrance layer around the immobilized lipase which may have limited the access of the hydrophobic substrate to enzyme active site. This finding has also been shown when long-chain triglyceride was modified with FA [17].

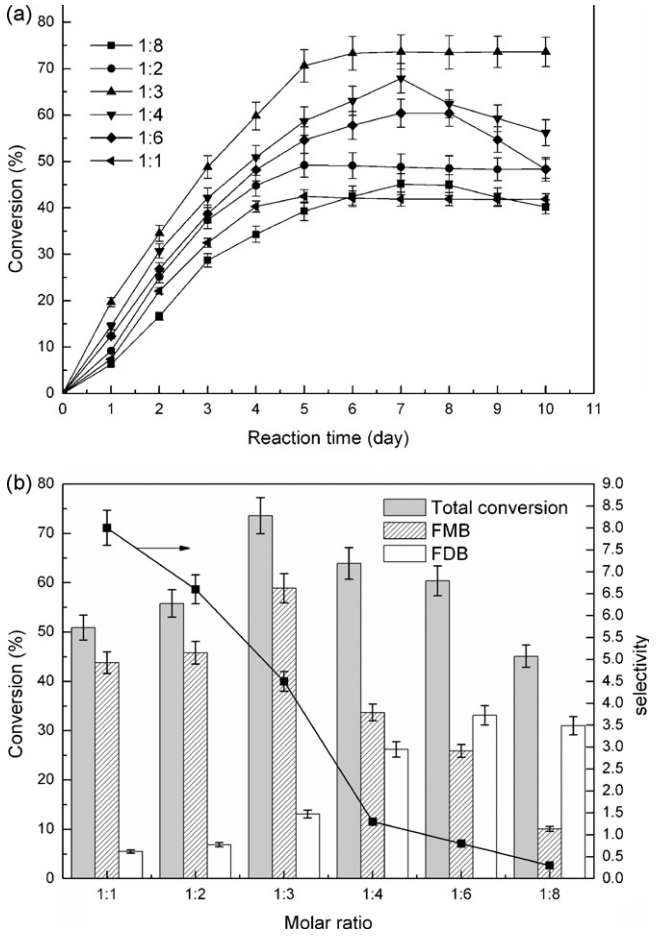

Fig. 2. Effect of molar ratio on lipase-catalyzed feruloylated lipids. (a) Conversion of reaction; (b) conversion of FDB, FMB and selectivity of different molar ratio at the maximum conversion of reaction. (Reaction conditions: toluene 3 mL, 50 °C, Novozym 435 120 mg, a_w 0.23, 210 rpm.)

The effect of molar ratio on the selective formation of FDB and FMB at the maximum conversion of reaction is shown in Fig. 2b. The formation of FDB and FMB may be attributed to the dependence of the feruloylated lipid formation on the partial removal of the butyric acid moiety from TB to form DB and MB. The results indicated that the conversion of FDB increased significantly from 5.5% to 35% when decreasing the molar ratio of EF to TB from 1:1 to 1:8. However, the conversion of FMB increased from 43.8% to a maximum of 59.9% with a ratio of 1:3 and decreased with the lower ratio. Excess in TB seems to have more beneficial effect on the formation of FDB than that of FMB. Thus, the reaction selectivity decreased with the increasing concentration of TB. This phenomenon has also been reported by Dossat et al. for the lipase-catalyzed transesterification of high oleic sunflower oil with butanol [21].

3.5. Influence of reaction temperature

Reaction temperature has a significant influence on the activity, selectivity and stability of a biocatalyst. Higher temperature can activate the substrate molecules, reduce the viscosity of reaction and lead to a higher reaction rate. However, higher temperature will also lead to greater lipase deactivation. Therefore, an optimal temperature should be selected in terms of the overall performance of the reaction. The effect of temperature on the total production was investigated (Fig. 3a). Present results show that in the range of 40–50 °C, the rate of reaction and total conversion increased with increasing temperature. However, higher temperature caused a significantly drop of both the reaction rate and total conversion. This may be due to partial lipase deactivation at higher temperature. The optimal temperature 50 °C for lipase-catalyzed EF with TB falls within the range of 45–60 °C, which reported in the literature for Novozym 435-catalyzed transesterification between selected phenolic acid with aliphatic molecule [22].

The effect of temperature on selective synthesis of FMB and FDB at the maximum conversion of reaction is shown in Fig. 3b. The results indicated that the conversion of FMB and FDB increased from 33.7% and 4.5% to 73.6% and 13.6% respectively with increasing the temperature from 40 to 50 °C; whereas, both the conversion of FMB and FDB decreased by further increasing temperature. For all temperature tested, the decreased selectivity of the process was observed with increasing temperature. These finding suggested that high temperature have had an inhibitory effect on the hydrolysis of TB, the rate of FMB formation is slower than FDB, which leads to the decrease in selectivity.

3.6. Effect of agitation speed

In order to evaluate the influence of agitation speed on the conversion and selectivity, various speeds were used at 50 °C for 7 days (Fig. 4a). As can be seen the rate of reaction and total conversion increased with increasing speeds from 150 to 210 rpm. However, as the speed was above 210 rpm, the conversion yield decreased. It was observed that substantial catalyst particles were thrown outside the liquid phase at 210 rpm, sticking to the wall of the reactor, which would reduce the effective catalyst loading. Further, it may also be due to the inactivation of the enzyme caused by foam formed at high speeds.

Fig. 4b depicts the effect of agitation speed on the selectivity synthesis of FDB and FMB at the maximum conversion of reaction. The conversion of FMB and FDB is increased from 25.8% and 5.9%

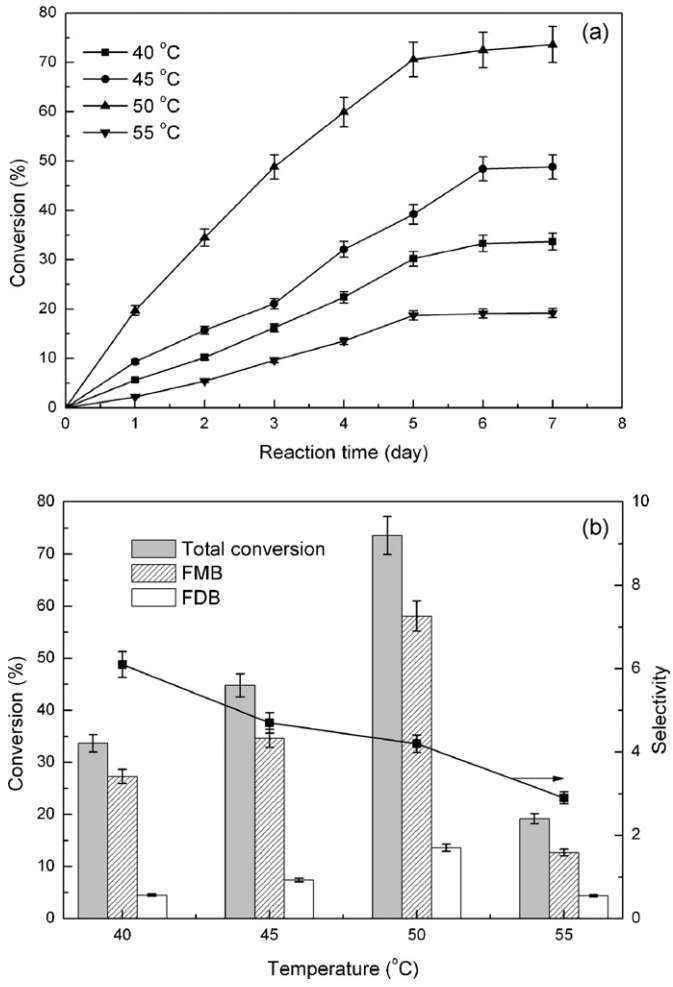

Fig. 3. Effect of temperature on lipase-catalyzed feruloylated lipids. (a) Conversion of reaction; (b) conversion of FDB, FMB and selectivity of different temperature at the maximum conversion of reaction. (Reaction conditions: toluene 3 mL, EF 0.5 mmol, TB 1.5 mmol, Novozym 435 120 mg, a_w 0.23, 210 rpm.)

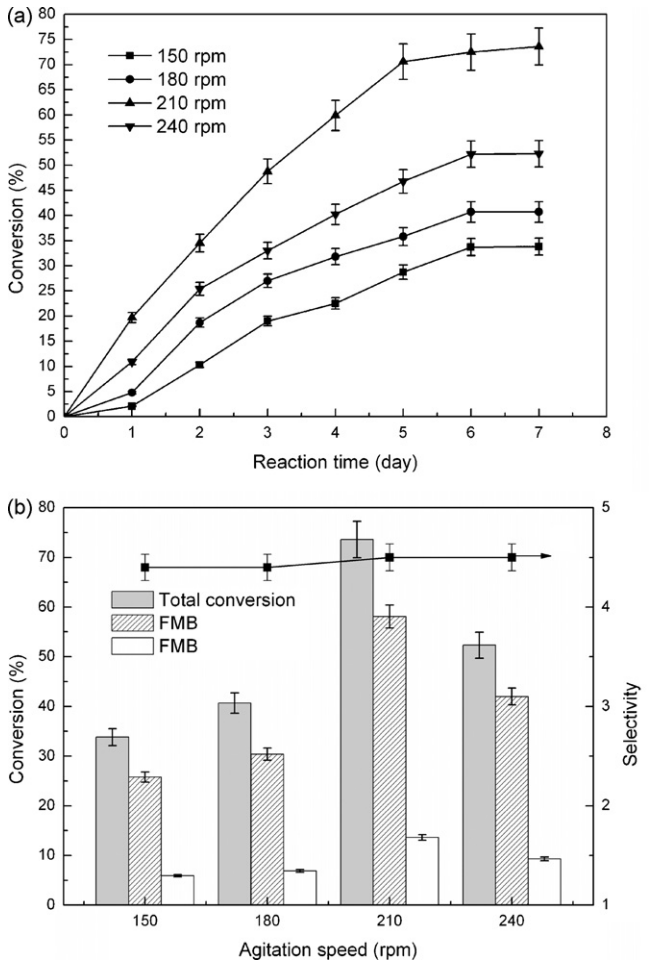

Fig. 4. Effect of agitation speed on lipase-catalyzed feruloylated lipids. (a) Conversion of reaction; (b) conversion of FDB, FMB and selectivity of different agitation speed at the maximum conversion of reaction. (Reaction conditions: toluene 3 mL, EF 0.5 mmol, TB 1.5 mmol, Novozym 435 120 mg, a_w 0.23, 50 °C.)

to 58.1% and 13.6% respectively with increasing the agitation speed from 150 to 210 rpm; whereas, both the conversion of FMB and FDB sharply decreased when the speed increased to 240 rpm. It was notable that although the yield of the product FDB and FMB was different respectively at various agitation speeds, the selectivity of reaction remained relatively constant. This suggests that agitation speed has almost no impact on the selectivity of reaction.

3.7. *Influence of water activity*

Transesterification reactions do not involve water. Nevertheless, the control of water is important as it is associated with the formation and maintenance of enzyme's active conformation or the "loosening up" of the rigid structure of an enzyme [23]. On the other hand, an excess of water in reaction media will inhibit the synthetic reaction while promoting the hydrolysis of the acylated products [24] and the acyl donors [25]. It was observed that lipase-catalyzed transesterification of EF and TB had a clear a_w dependence. The moderate optimal water activity ($a_w < 0.23$) could be rationalized by the ambivalent effects of water on the lipase-catalyzed synthetic reactions in organic media (Fig. 5a). When a_w was 0.33 there was also a fast reaction rate at initial 5 days, but unfortunately, the conversion and reaction rate started to obviously go down thereafter. This phenomenon was also observed by Chang et al. [26]. The reaction rate and conversion became much lower by changing a_w from 0.33 to 0.75. This was because hydrolysis of reaction would be favored due to the abundance of water in this reaction system ($a_w > 0.55$). The strong competing hydrolysis led to much lower transesterification extent at high water activities.

The effect of a_w on the selective synthesis of FDB and FMB at the maximum conversion of reaction is shown in Fig. 5b. The results showed that the conversion of FMB increased with increasing a_w and reached its maximum when a_w was 0.33, and then decreased by further increasing a_w. However, the conversion of FDB decreased obviously with increasing a_w. In the whole process, the selectivity of reaction increased with increasing a_w value. It could be attributed to that a relatively high water activity is in favor of hydrolysis of TB and therefore, represses FDB formation compared to FMB, but too high water activity ($a_w > 0.55$) can promote hydrolysis of both substrates and products which lead to the decrease in conversion yield of both FDB and FMB.

It is concluded that water activity had an impact on both reaction conversion and selectivity. This finding is inconsistent with the report by Laszlo and Compton who indicated that a_w had minimal impact on transesterification EF with soybean oil [12]. One of the probability causes of this difference was that the short-chain substrate TB is more water soluble and thus react differently than long-chain substrate in non-aqueous system. In addition, different chain of free acids in the reaction system at various a_w might also be contributing factors.

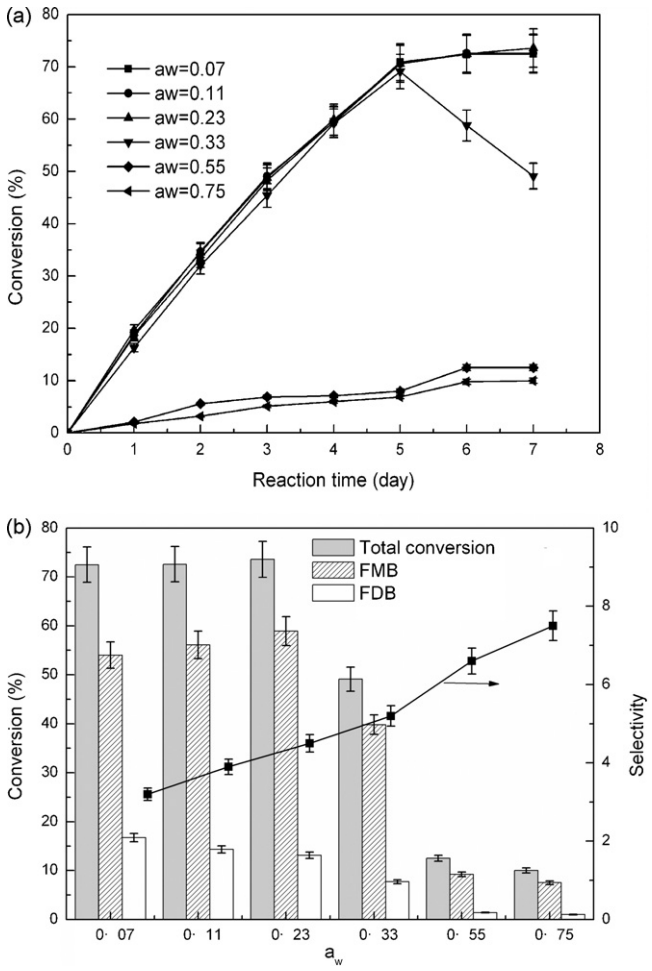

Fig. 5. Effect of a_w on lipase-catalyzed feruloylated lipids. (a) Conversion of reaction; (b) conversion of FDB, FMB and selectivity of different a_w at the maximum conversion of reaction. (Reaction conditions: toluene 3 mL, EF 0.5 mmol, TB 1.5 mmol, Novozym 435 120 mg, 210 rpm, 50 °C.)

3.8. Operational stability of the lipase

One of the limitations associated with the industrial application of enzymes is their high cost and instability under operational conditions [27]. Thus assay reusability after each operation is essential. After completion of the reaction, the lipase particles was filtered off and then added into fresh reaction medium directly to catalyze a second run. The lipase-catalyzed reactions were repeated 14 times and we found that there was no significant loss of lipase activity (Table 3). Even at 14th consecutive repetitive batch of reaction, the conversion was slightly decreased to 65%, and the conversion yield of FMB and FDB was still maintained 54.7% and 8.3% respectively. This excellent performance of the Novozym 435 might have benefited from the relatively mild operation conditions used. The solubility of substrates in organic medium appears to also help enzyme to retain its activity. These results indicated the industrial potential of the operation protocol developed in this work.

Table 3
The conversion yield of total reaction, FDB and FMB after each run operation.

Run	The yield of product (%) ± S.D.		
	Total yield	FDB yield	FMB yield
1	73.6 ± 1.1	13.1 ± 1.3	58.3 ± 1.9
2	73.2 ± 1.3	12.9 ± 1.9	58.1 ± 1.2
3	73.2 ± 0.7	12.6 ± 2.1	58.1 ± 1.1
4	73.1 ± 1.5	12.3 ± 1.2	57.7 ± 1.7
5	72.9 ± 0.9	12.1 ± 1.5	58.1 ± 1.3
6	72.1 ± 1.2	11.7 ± 1.8	57.8 ± 1.8
7	72.2 ± 1.1	11.6 ± 1.1	57.8 ± 1.5
8	71.7 ± 1.6	11.5 ± 1.4	56.8 ± 0.6
9	70.6 ± 1.3	11.2 ± 1.5	56.7 ± 1.1
10	69.3 ± 1.9	10.4 ± 1.7	56.1 ± 1.7
11	68.1 ± 2.1	10.1 ± 2.2	55.9 ± 1.6
12	67.3 ± 1.3	9.5 ± 1.2	55.7 ± 2.2
13	66.9 ± 1.4	9.1 ± 1.6	55.1 ± 1.7
14	65.4 ± 1.7	8.3 ± 2.1	54.7 ± 1.2

Reaction conditions: toluene 3 mL, EF 0.5 mmol, TB 1.5 mmol, Novozym 435 120 mg, a_w 0.23, 50 °C.

4. Conclusions

In the present study, we described a facile enzymatic synthesis approach for the preparation of feruloylated-structured lipids through the transesterification of ethyl ferulate and tributyrin in toluene. The structural analysis (NMR, ESI-MS) confirmed the formation of two major feruloylated lipid products. The influencing factors including organic media, enzyme source, molar ratio, reaction temperature, agitation speed and water activity were systematically investigated. Meanwhile, the effect of operational condition on distributions of FA on the backbone of TB was also studied. There was no evident loss in lipase activity and stability after being repeatedly used for 14 runs. Further studies are in process to compare pharmacological capacity of non-modified native molecule and different structure of lipophilized derivatives. It could provide a better understanding between pharmacological capacity measured by various assays and physicochemical descriptors including steric and hydrophobic effects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2008.11.005.

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