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Lipase-catalyzed synthesis of feruloyl oleins in solvent-free medium

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ABSTRACT

Lipase-catalyzed transesterification of ethyl ferulate (EF) with triolein (TO), to form feruloyl oleins in a solvent-free medium, was investigated. Water activity (a_w) had an obvious influence on transesterification efficacy. A possible explanation is that the reaction of EF with TO would be deacylation-rate determined. In order to enhance the combined yield of feruloyl diolein (FDO) and feruloyl monoolein (FMO) and minimize hydrolysis of substrate, glycerol (rather than water) was introduced to the reaction. A maximal combined yield of 29.0% of FDO and FMO was obtained using a 1:3 mole ratio of glycerol to TO in solvent-free medium at 60 °C. Compared to the feruloyl olein synthesis conducted by the normal pressure magnetic stirring procedure, the synthesis of feruloyl oleins by a vacuum-rotary evaporation procedure, with a vacuum of 7.5 mmHg, affords higher yields within the same time. The enzyme can be reused several times without significant loss of activity.

1. Introduction

Synthetic antioxidants have been used in foods over many decades; however, there are growing concerns about their carcinogenic capabilities (Shahidi & Naczk, 1995). Ferulic acid (4-hydroxy-3-methoxy cinnamic acid, FA) is a natural antioxidant with potential health benefits against cardiovascular problems, inflammatory diseases and cancer (Ho, 1992; Silva et al., 2000). Ferulic acid has been found in higher plants and has long been studied for its health-promoting properties, partially owing to its capacity to inhibit oxidation (Castelluccio, Bolwell, Gerrish, & Rice-Evans, 1996). Due to exhibiting a powerful antioxidation, ferulic acid and its derivatives are well-known antioxidants in foods (Warner & Laszlo, 2005). For example, ferulic acid esters have been shown to inhibit deterioration in frying (Kochar, 2000).

However, ferulic acid is a small and polar compound with only a limited solubility in oils. The low solubility of ferulic acid in hydrophobic medium reduces its antioxidant effectiveness in inhibiting autooxidation of fats and oils (Schuler, 1990; Stamatis, Sereti, & Kolisis, 2001).

The strategy of esterification of hydrophilic ferulic acid with lipophilic molecules, such as aliphatic alcohols, can be employed to alter its solubility in a hydrophobic medium. It has been found that the hydrophobic derivatives, alkyl ferulates, have a higher antioxidative activity than has unmodified ferulic acid for the prevention of oxidation of linoleic acid in a bulk system (Fang, Shima, Kadota, Tsuno, & Adachi, 2006).

Chemical synthesis of these derivatives is difficult, as ferulic acid is heat-sensitive and susceptible to oxidation under certain pH conditions (Guyot, Bosquette, Pina, & Graille, 1997). Environment-friendly preparation of hydrophobic derivatives of ferulic acid is therefore a challenge for researchers. Enzymatic direct esterifications of ferulic acid with aliphatic alcohols have been reported (Buisma et al., 1998; Compton, Laszlo, & Berhow, 2000; Guyot et al., 1997; Stamatis, Sereti, & Kolisis, 1999; Stamatis et al. 2001; Yoshida, Kimura, Kadota, Tsuno, & Adachi, 2006). However, the reaction rate for the condensation of ferulic acid with an alcoholic substrate is low because ferulic acid has conjugation with a carboxyl group and a bulky noncarboxylic region (Kobayashi, Adachi, & Matsuno, 2003). Transesterifications of ethyl ferulate with vegetable oil, catalyzed by *Candida antarctica,* were also reported (Compton & King, 2001; Compton, Laszlo, & Berhow, 2006; Laszlo, Compton, Eller, Taylor, & Isbell, 2003). The enzyme-catalyzed transesterification can produce a mixture of feruloyl monoacylglycerol and feruloyl diacylglycerol that were lipophilic antioxidants. Previous studies have shown that enzymatic syntheses of these lipids have low time efficiencies, and specific solvent requirements. Recently, a novel enzymatic route for synthesis of feruloyl monoacylglycerol and feruloyl diacylglycerol has been reported (Sun et al., 2007). The reaction is catalyzed by immobilized *C. antarctica* lipase

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B. Feruloyl monoacylglycerol and feruloyl diacylglycerol were synthesized in a two-step reaction: ethyl ferulate was first transesterified with glycerol and then this was esterified with oleic acid. The yields of the combined feruloyl monoacylglycerol and feruloyl diacylglycerol in the second reaction reached 96%. However, in this synthesis route, high glycerol/ethyl ferulate ratios are employed and excessive quantities of nonreacted glycerol are found. Nonreacted glycerol has a crucial effect in following lipase-catalyzed esterification of oleic acid with polar feruloyl glycerol. Also, at higher glycerol/ethyl ferulate ratios, the immobilized lipase and EF frequently agglomerate, resulting in significant activity loss.

Laszlo et al. have previously reported a facile two-step process: soybean oil glycerolysis, followed by ethyl ferulate transesterification (Laszlo & Compton, 2006). It has been found that the monoacylglycerol and diacylglycerol from soybean oil glycerolysis are much more reactive than is soybean oil during ethyl ferulate transesterification, which leads to greater reactor productivity. However, for commercial considerations, introducing additional processing steps to generate monoacylglycerol and diacylglycerol are uneconomical.

In this paper, ethyl ferulate (EF), triolein (TO) and glycerol were reacted synchronously. Glycerol was adsorbed onto silica, prior to the introduction of EF, TO and lipase, to avoid fouling the lipase and its support. The aim was to develop a biotechnological process for the production of feruloyl oleins in a solvent-free medium. A vacuum-rotary evaporation procedure for enzymatic synthesis of feruloyl diolein (FDO) and feruloyl monoolein (FMO) from EF and TO was successfully carried out. By using the vacuum-rotary evaporation procedure, feruloyl oleins have been produced with higher yield and the enzyme can be reused several times without significant loss of activity.

2. Materials and methods

2.1. Chemicals and enzyme

Ferulic acid (FA, purity > 99%) and ethyl ferulate (EF, purity > 99%) were from Suzhou Chang Tong Chemical Co., Ltd (Suzhou, China). Triolein (TO, purity > 98%) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other regents were of chemical purity. Novozym 435 (*C. antarctica* lipase immobilized on polyacrylic resin, with an activity of 10,000 propyl laurate units, PLU, g^{-1} solid enzyme) was purchased from Novozymes A/S (Bagsvaerd, Denmark). Thin-layer chromatography (TLC) plates were purchased from Merck (Darmstadt, Germany).

2.2. General procedure for enzymatic transesterification

The enzymatic syntheses of feruloyl oleins were performed without solvent. To conduct the reaction under the neat conditions, a 1:2 EF/TO molar ratio is needed to provide enough solution volume (TO volume) to dissolve solid EF and to stir the suspended immobilized enzyme. However, a lower EF/TO ratio reduces the concentration of the feruloyl monoolein (FMO) and feruloyl diolein (FDO) formed in the final reaction mixture. To guarantee the yield and concentration of feruloyl oleins (FMO and FDO), a 1:2 EF/TO molar ratio was employed in this study.

Transesterification reactions were conducted in 25 ml capped conical flasks. A typical reaction mixture contained EF (1.0 mmol), TO (2.0 mmol), and glycerol (0.0–1.0 mmol). Varying amounts of glycerol were first blended with 90 mg of silica gel in the 25 ml capped conical flasks. Glycerol-adsorbed to silica was added prior to the introduction of EF, TO and lipase, to avoid fouling of the immobilized lipase. EF (1.0 mmol) was dissolved in TO (2.0 mmol) to give a clear, colourless and viscous solution at temperatures from 45 °C to 75 °C. The transesterification was initiated by the addition of 110.0 mg of the immobilized lipase (Novozym 435) into each capped conical flask. Flasks were placed, upright, on a magnetic stirrer (200 rpm) and incubated. After the completion of reaction, lipase was filtered off and washed thoroughly with hexane (a_w < 0.01) and reused in the reusability study. Control experiments, without lipase, were also carried out in tandem with these trials. These standard conditions were used throughout the experiments except when otherwise stated in the text. Samples of 100 μl were periodically taken for analysis. All the assays were run in duplicate.

2.3. Vacuum-rotary evaporation procedure for enzymatic transesterification

Reactions were conducted in 25 ml round-bottom flasks on a vacuum-rotary evaporator. Silica (90 mg) and glycerol (0.67 mmol) were mixed together, and then EF (1.0 mmol), TO (2.0 mmol) and Novozym 435 (110 mg) were added. The flasks were incubated at different temperatures and 200 rpm under 7.5 mmHg (0.001 MPa) vacuum pressures. At the end of reaction, the recovery and reuse of immobilized lipase were subjected to the general procedure for enzymatic transesterification (*vide supra*).

2.4. Water activity pre-equilibration of reaction medium

Water activity is an important consideration for biocatalysis in nonaqueous medium. Water activity or aw is a measurement of the energy status of the water in a system. It is defined as the vapour pressure of water divided by that of pure water at the same temperature. Before the start of the reaction, the enzyme and the substrates (TO, EF and glycerol; EF was dissolved in TO and glycerol was adsorbed onto silica) were pre-equilibrated for at least 3 d in separate sealed containers enclosed with saturated salt solutions or solid adsorbent to establish fixed water activities for transesterification. Pre-equilibration was done at 25 °C. The solid adsorbent was 3 Å molecular sieves (a_w < 0.01). The saturated salt solutions used were prepared with LiBr (a_w: 0.05), LiCl (a_w: 0.11), CH_3COOK (a_w: 0.23), $(MgNO_3)\cdot 6H_2O$ (a_w: 0.54), NaCl (a_w: 0.75), KCl (a_w: 0.85), K_2Cr_2O7 (a_w: 0.98) (Wehtje, Costes, & Adlercreutz, 1997).

2.5. Monitoring and analysis of reaction mixtures

In order to monitor the reaction progress, samples of 100 μl were withdrawn at intervals. Samples were diluted 1:10 (vol/vol) in acetone and analyzed by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC). Aliquots of samples and blank reaction components were monitored by TLC on silica gel 60 F_{254} plates with fluorescent indicators (Merck. KGaA, Darmstadt, Germany). The TLC migration was carried out with a solvent mixture of dichloromethane/benzene/ethyl ether/hexane (50:30:20:1, v/v/v/v). The TLC plates were visualized under UV. Results were estimated from intensity of spots on TLC. The bands of feruloyl monoolein (FMO), feruloyl diolein (FDO), ethyl ferulate (EF), ferulic acid (FA) and feruloyl glycerol (FG) were detected at UV light (254 nm). As expected, the FG migrated the slowest, while the feruloyl diolein migrated the fastest.

The transesterification reaction was also analyzed by HPLC (Waters Alliance 2695), following previously described procedures (Sun et al., 2007). Samples were diluted with acetone and further diluted 20-fold with methanol. FA, EF, FG, FMO and FDO were determined with a X-Terra C18 reversed phase column (5 μm, 150 mm × 2.1 mm), using a binary gradient of solvent A (99% water, 1% glacial acetic acid) and solvent B (100% methanol) at a flow rate of 1 ml/min. The elution sequence was a linear gradient

from 50% (v/v) B to 100% B in 30 min, followed by an isocratic flow of 100% B for 15 min at 35 °C. The eluate was monitored at 254 nm, using a variable wavelength UV detector. The sample injection volume was 10 μl.

According to previous reports (Compton & King, 2001; Compton et al., 2000), the yields were calculated, based on the total peak area of EF, FDO, FMO, FA and FG detected at 254 nm. The sum of all ferulate species peak areas remained constant over the time course of the reaction, allowing accurate FDO and FMO yields to be calculated as the percentage ratio of the FDO and FMO peak area to the total peak area of all ferulate species. FA and FG are two unwanted by-products.

3. Results and discussion

3.1. The target product of enzymatic transesterification

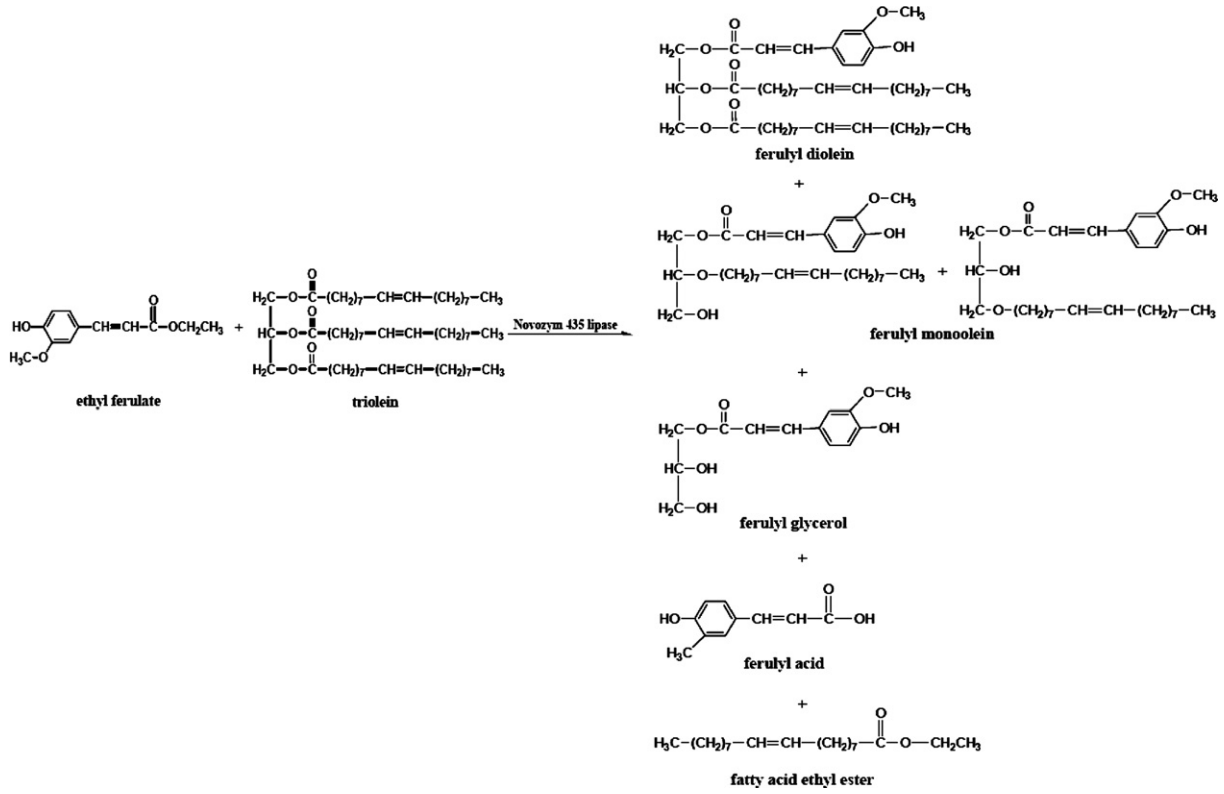
It was previously reported that transesterification of soybean oil with EF by immobilized lipase *C. antarctica* lipase B (Novozym 435) produced a multitude of feruloyl monoacylglycerols and feruloyl diacylglycerols. Additional products formed in relatively small quantities are FA, resulting from EF hydrolysis, and FG. Diferuloyl-substituted species have not been detected (Laszlo et al., 2003). Hydrophobic feruloyl monoacylglycerols and feruloyl diacylglycerols can be used as antioxidant ingredients. (Compton, Kenar, Laszlo, & Felker, 2007; Warner & Laszlo, 2005).

In this paper, TO was chosen as a representative triacylglycerol (TAG) for use in place of soybean oil to simplify the reaction products. Lipase-catalyzed transesterification of EF with TO in solvent-free medium was investigated. As shown in Scheme 1, Novozym 435 (*C. antarctica* lipase B)–catalyzed transesterification of EF and TO resulted in a mixture of FDO and FMO, These hydrophobic feruloyl oleins (FDO and FMO) can be used as antioxidant ingredients. Trace amounts of FA and FG, which resulted from the hydrolysis of EF and feruloyl oleins, are unwanted hydrophilic by-products and are not included as products. For this reason, yield percentage is based on a definition of product as comprising FDO and FMO species.

3.2. Influence of a_w on feruloylation kinetics

Efficiency of enzyme-catalyzed reaction in nonaqueous medium is under the control of a_w. Initially, lipase-catalyzed synthesis of feruloyl oleins was attempted without glycerol introduction. Under this condition, by equilibrating enzyme and substrates (i.e. TO and EF) with saturated salt solutions or 3 Å molecular sieves, the initial a_w for EF transesterification with TO can be fixed over a broad range from <0.01 to 0.75. The resulting transesterification efficacy, in function of a_w, is shown in Fig. 1. It was observed that a_w had an obvious influence on the transesterification efficacy. The rise in reactivity with increasing a_w can be observed here for the transesterification of EF with TO. Reaction under the lowest a_w condition (< 0.01, equilibrated with 3 Å molecular sieves) was slower than at the highest a_w (0.75). When a_w was changed from < 0.01 to 0.75, the combined yield of FDO and FMO increased from 7.6% to 15.5%. However, the yield of FDO decreased slightly from 10.0% to 9.4% when a_w was changed from 0.66 to 0.75.

A possible explanation for this result is that the transesterification of EF with TO would be deacylation-rate determined. As shown in Scheme 2, the transesterification of EF is dependent upon the removal of oleate group from triolein, to form monoolein and diolein, which then can react with the feruloyl-lipase intermediate to form FMO and FDO. If the transesterification is rate-limited by the formation of acyl acceptor, the rates of formation of monoolein and diolein may control the reaction. A combined yield of FDO and FMO increasing with a_w may arise from the generation of small



Scheme 1. Novozym 435-catalyzed transesterification of ethyl ferulate and triolein Feruloyl group substitution at the glycerol sn-1 position is shown for simplicity as the regioselectivity of the reaction is unknown.

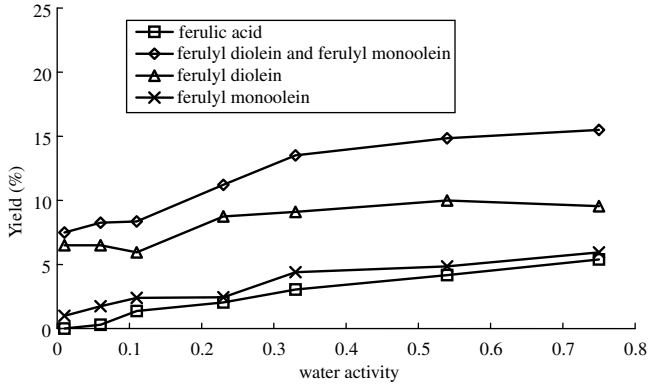
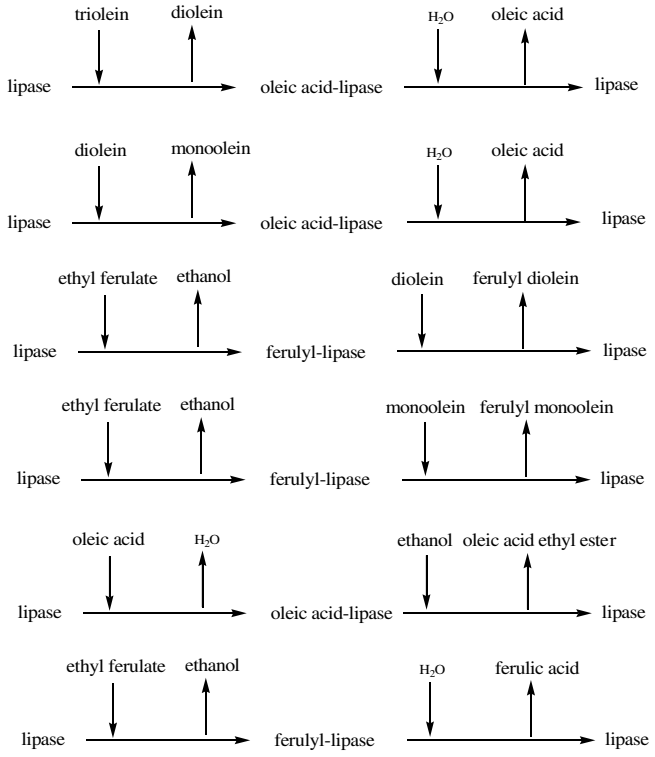


Fig. 1. Relationship between initial water activity (a_w) and transesterification efficacy. Reaction conditions: 1.0 mmol EF, 2.0 mmol TO, temperature 60 °C, 110.0 mg Novozym 435 lipase, magnetic stirrer speed 200 rpm, and reaction time 72 h.



Scheme 2. The sequential reaction sequence of transesterification of ethyl ferulate with triolein.

amounts of monoolein and diolein by hydrolysis. This finding is consistent for the transesterification of EF with soybean oil in a sequential process of triglyceride hydrolysis, followed by a feruloylation reaction via a Ping–Pong Bi–Bi mechanism (Xu, 2000).

However, as shown in Fig. 1, at a_w 0.11, detectable quantities of FA were produced during EF transesterification with TO, although the amount of EF hydrolysis was less than 2% within the reaction time of 72 h. EF hydrolysis generally increased with a_w.

3.3. Glycerol effects on feruloylation kinetics

Under the lowest a_w condition (a_w < 0.01, equilibrated with 3 Å molecular sieves), attempts to react EF and TO together, using *C. antarctica* lipase, produced a mixture of FMO and FDO. FA and FG, which resulted from the hydrolysis of EF and feruloyl oleins, have not been detected. However, the combined yield of FDO and FMO reached only 7.6% after 72 h (Fig. 2).

As shown above, since water can cause the hydrolysis of substrates, the enhancement of a_w, to increase the combined yield of FDO and FMO, was not attempted. Instead, to enhance the combined yield of FDO and FMO, we attempted to introduce glycerol into the transesterification of EF with TO and react EF, TO and glycerol synchronously. In these experiments, glycerol was adsorbed onto silica, prior to the introduction of enzyme, to avoid the agglomeration of the enzyme with excess glycerol, a common practice in lipase-catalyzed preparation of diacylglycerol and monoacylglycerol from triolein (Rendón, López-Munguaía, & Castillo, 2001; Weber & Mukherjee, 2004). As shown in Fig. 2, the introduction of silica-adsorbed glycerol, in the transesterification of EF with TO, enhanced the combined yield of FDO and FMO. The combined yield of FDO and FMO after 72 h with 1:3 glycerol/TO was 29.0% and almost fourfold higher than that in the absence of glycerol. A possible explanation for this result is consistent with the above explanation for the transesterification using EF and TO as initial substrates. The transesterification of EF with TO would be deacylation-rate determined. The enhanced combined yield of FDO and FMO found can be attributed to the generation of greater amounts of monoolein and diolein by glycerolysis and the partially deacylated diolein and monoolein react with EF more quickly than does TO (Scheme 3).

However, at a higher glycerol/TO ratio (1:2 glycerol/TO), due to the large quantity of glycerol that is available to equester ferulyl groups to form FG, detectable quantities of FG, an unwanted water-soluble product, were formed during EF transesterification with TO. Also, EF precipitation (induced by excess solubilized glycerol) could be found at 1:2 glycerol/TO ratios, and the immobilized enzyme frequently agglomerated, resulting in significant decline of the combined yield of FDO and FMO. So, operating under these conditions, with 1:3 glycerol/TO mole ratios, was implemented in the following experiments.

Fig. 2 also shows that the Novozym 435-catalyzed EF/TO transesterification without glycerol produced the highest FDO to FMO ratio. This may be explained as follows: The introduction of glycerol into the mixture of EF and TO increase the polarity of the system. The thermodynamic properties of polarity systems were propitious to forming polar product. FDO is less polar than FMO; as a result, it

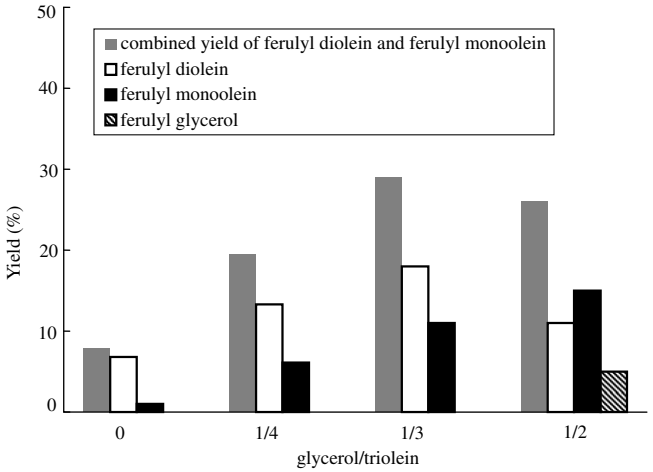
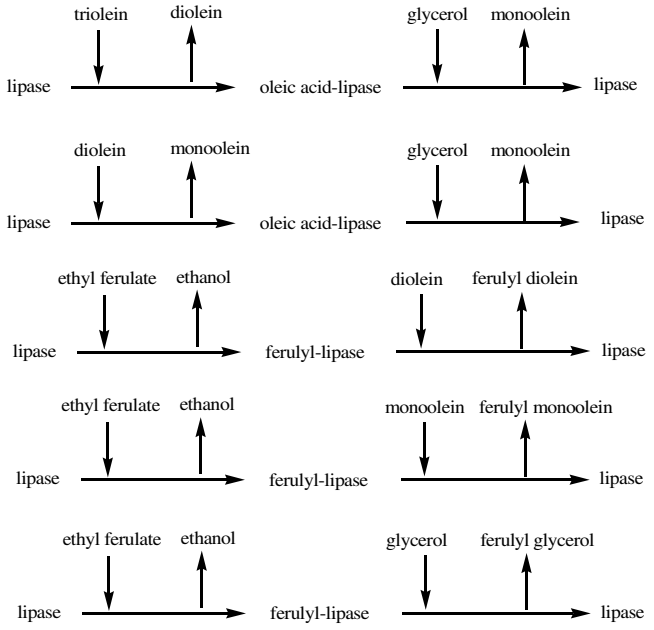


Fig. 2. Transesterification of ethyl ferulate with triolein in the absence or presence of glycerol. Reaction conditions: a_w < 0.01, 1.0 mmol EF, 2.0 mmol TO, 0.0–0.1 mmol glycerol, temperature 60 °C, 110.0 mg Novozym 435 lipase, magnetic stirrer speed 200 rpm, and reaction time 72 h.



Scheme 3. The sequential reaction sequence of transesterification using ethyl ferulate, triolein and glycerol as initial substrates.

is more soluble in the non-polar EF/TO system. Therefore, increasing the amounts of the glycerol in the EF/TO mixture has favoured shifting of the thermodynamic equilibrium toward synthesis of the less hydrophobic FMO.

3.4. Vacuum-rotary evaporation procedure for enzymatic transesterification batch reactions

In the above general procedure for enzymatic transesterification, a maximal combined yield of 29.0% of FMO and FDO was obtained, using a 1:3 mole ratio of glycerol to TO at 72 h (Fig. 2). The low reaction yield was probably the result of inefficiency of ethanol removal. Also, we observed that, in the solvent-free system, difficulties in mechanical mixing, owing to the high viscosity of substrate mixtures, occurred. To create an efficient reaction system, the reaction was performed using a vacuum-rotary evaporation procedure (7.5 mmHg, 200 rpm. The yield rises with the enhancement in mixing effect. The rotation speeds between 150 and 200 rpm had minor effects on the combined yield of FMO and FDO). As shown in Fig. 3, the vacuum-rotary evaporation procedure was found to give high combined yield of FMO and FDO. The combined yield of FMO and FDO increased sharply up to 48 h to reach 54.3%, and then increased smoothly up to 72 h to reach 59.5%, which is much faster than the normal pressure magnetic stirring procedure.

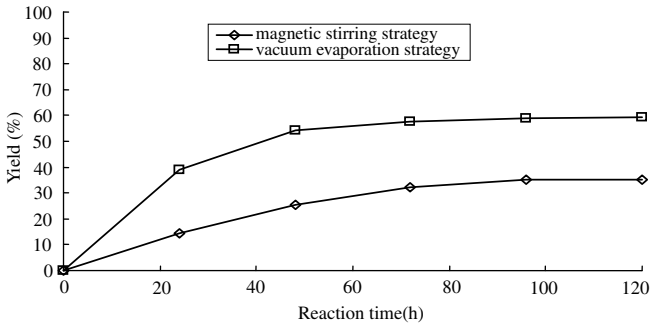


Fig. 3. Time course for transesterification of EF with triolein by normal pressure magnetic stirring procedure and vacuum evaporation procedure. Reaction conditions: a_w < 0.01, 1.0 mmol EF, 2.0 mmol TO and 0.67 mmol glycerol, temperature 60 °C, 110.0 mg Novozym 435 lipase, magnetic stirrer or rotation speed 200 rpm.

These results may be attributed to elimination of external mass transfer resistance and high efficiency of ethanol removal. The vacuum-rotary evaporation procedure is able to create an effective interaction among the different phases of the enzymatic reaction of EF, TO, glycerol and to eliminate the external mass transfer resistance. Also, the vacuum-rotary evaporation procedure minimizes the negative effects of resulting ethanol on lipase activity and shifting of the reaction towards the synthesis of FMO and FDO.

Compared to the FMO and FDO synthesis conducted by the normal pressure magnetic stirring procedure, the vacuum-rotary evaporation procedure affords a higher combined yield of FMO and FDO within a shorter period of time. Therefore, the vacuum-rotary evaporation strategy was our choice in the following experiments.

3.5. The effect of temperature on the syntheses of ferulyl oleins

Reactions were conducted in 25 ml round-bottom flasks at different temperatures and 200 rpm under 7.5 mmHg (0.001 MPa) vacuum pressures. The maximum combined yield of FMO and FDO (59.5%) is achieved at temperatures between 60 °C and 65 °C. The combined yield was 43.1% at 45 °C and 57.9% at 70 °C. The significant increase of yield with increasing temperature from 45 °C to 65 °C results from the acceleration of diffusion and intrinsic enhancement of enzyme activity. On the other hand, the decreasing viscosity of the reaction system and high efficiency of ethanol removal are also believed to contribute to the increase of reaction yield. However, high reaction temperature may cause deactivation of enzyme. According to the Novozym 435 product sheet, Novozym 435 is a heat-tolerant, immobilized enzyme with a maximum activity at 70–80 °C. But it is suggested that the enzyme should be used at 40–60 °C for the sake of its stability. In this work, the reusability study of Novozym 435 was carried out at 60 °C for ferulyl oleins synthesis.

3.6. Batchwise operation

When the enzymatic repetitive batch transesterifications were conducted by the vacuum-rotary evaporation procedure, the combined yield of FDO and FMO was 59.0% at 72 h. The recovered immobilized lipase in the present work can be used nine times without significant loss of activity. Even at the 9th consecutive repetitive batch of reaction, the combined yields of FDO and FMO were still up to 56.0%. Excellent performance of *Candida antarctica* lipase might benefit from the relatively mild conditions used. The occurrence of ethanol-deprived medium in the solvent-free system seems to help the lipase to retain its activity. Although a general normal pressure magnetic stirring procedure was not the aim of this work, the procedure was also tested to demonstrate the advantages of the vacuum-rotary evaporation procedure. When the enzymatic transesterifications were conducted by the general normal pressure magnetic stirring procedure, the yield decreased as the reaction proceeded and the recovered immobilized lipase lost almost all of its activity at the 8th consecutive repetitive batch of reaction. This is believed to result from inefficiency of ethanol removal. These results suggest that the vacuum-rotary evaporation procedure was very efficient for synthesis of ferulyl oleins. Therefore, the transesterification of EF with TO in the presence of glycerol by vacuum-rotary evaporation batch reaction is a viable procedure for ferulyl oleins synthesis that could be used for antioxidative ingredients in oil formulations.

4. Conclusion

a_w had an impact on transesterification efficacy. The rise in reactivity with increasing a_w can be observed here for the transesterification of EF with TO. This result may be explained in that the reaction of EF with TO would be deacylation-rate determined, i.e. the transesterification of EF is dependent upon the removal of oleate groups from TO to form monoolein and diolein. A combined yield of FDO and FMO, increasing with a_w, may arise from the generation of small amounts of monoolein and diolein by hydrolysis. However, additional water is deleterious in this reaction. The water activity should be kept as low as possible.

In order to enhance the yield of ferulyl oleins, glycerol was introduced to the reaction and enhanced combined yields of FDO and FMO were found. In order to eliminate the external mass transfer resistance and remove ethanol produced, the vacuum-rotary evaporation procedure was used and gave high combined yield of FDO and FMO. Vacuum-rotary evaporation was very efficient for ethanol removal to shift the reaction in the desired direction. The solvent-free vacuum-rotary evaporation procedure established in this study provides an efficient means to synthesize FDO and FMO with a combined yield of 59.5% at 72 h. The enzyme can be reused several times without significant loss of activity. These results indicate the industrial potential of the operation protocol developed in this work.

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