Ultrasound-Assisted Enzymatic Synthesis of Fructose Oleic Ester using Supersaturated Fructose Solution

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ABSTRACT

Fructose solubility is one of the major challenges inhibiting enzymatic synthesis of fatty acid sugar ester. Therefore, this study aims to define the optimal parameters for manufacturing fructose oleic ester (FOE) utilizing a supersaturated fructose solution coupled with ultrasound technology. Factors such as esterification time, ultrasound power, and substrate flow rate were evaluated. The reaction was carried out by adding the supersaturated fructose solution and oleic acid to the immobilized lipase in the jacketed fluidized bed reactor equipped with an ultrasound. FOE was evaluated based on ester concentration, ester bond, and emulsification properties. The results showed thatesterification activity of lipase was 36.45±9.95 U/g matrix. Fructose concentration in the supersaturated fructose solution was 12.30±2.33 mg/mL. The optimal parameters for synthesizing FOE were defined at 180 Watt and 0.2 mL/min for 180 min of reaction after one time using a series of esterification apparatus. FOE concentration was 85.13±9.56% and the sample with the best conditions had Rf value of 0.2 to ~0.8, wave absorption band for ester group (C=O) at wavenumber ~1712 cm^{-1} with a new peak (C-O bond) at 1373 cm^{-1}, emulsion capacity 99.86±0.01%, emulsion stability of 99.29±0.04%, and droplet size of 1.00 µm with non-uniform droplet size distribution (polydispersity index (PDI)=1.00).

Keywords: Enzymatic esterification; fluidized bed reactor; oleic acid; supersaturated fructose solution; ultrasound

INTRODUCTION

Sugar fatty acid ester are non-toxic, tasteless, and possess high emulsion capacity and stability (Casas-Godoy et al., 2016), leading to the wide use as emulsifiers in food products. Fructose oleic ester (FOE) can reduce surface tension better than other sugar fatty acid ester (Ye & Hayes, 2012). Synthesis of FOE is carried out enzymatically using immobilized lipase. In general, enzymatic synthesis of FOE uses sugar crystals and oil as substrates. Given that sugar solubility in oil is low, the amount of dissolved sugar is small. The principle of enzymatic reaction requires a contact between substrates and enzyme active site, which affects the reaction rate. Therefore, the dissolved sugar must be able to access enzyme active site. The concentration of dissolved sugar also needs to be high for increased contact.

Supersaturated sugar solutions can be used as an alternative to increase the amount of dissolved sugar (Sang et al., 2008). These solutions contain solutes higher than the equilibrium concentration. The liquid and homogeneous form is easily transported or diffused into the active site of enzyme, thereby increasing the contact between substrate and enzyme. Polar fructose requires polar solvents, hence, tert-butanol can be used

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to dissolve fructose in the preparation of supersaturated fructose solutions. Tert-butanol is also used to dissolve fructose in oleic acid.

Another weakness of enzyme-catalyzed production of fatty acid sugar ester is the long reaction time, ranging from 24 h (Pappalardo et al., 2017; Liu, 2017) to 48 h (Hidayat et al., 2016). Ultrasound using high-frequency acoustic waves can be used as an alternative to improve the reaction because it increases enzyme activity by opening the lid lipase (More et al., 2017). Consequently, the transport of substrate to the active site of enzyme and product release increased (Lee et al., 2008) along with the reaction time. The high ultrasound power leads to higher contact of substrate with enzyme, increasing ester concentration in a shorter time. However, extremely high power may reduce enzyme activity due to damage or enzyme inactivation and substrate aggregation.

FOE synthesis requires a reactor for the catalyst, which can accelerate the reaction, such as fluidized bed reactor. In this reactor, the column is not filled with enzyme matrix to allow fluidization when substrate flows at a certain rate in the column, which is different from the packed bed reactor. Additionally, the substrate flow rate in the reactor contributes to the length of contact with enzyme active site.

Based on the description above, this study aims to determine the optimal parameters for the enzymatic synthesis of FOE, employing immobilized lipase within a fluidized bed reactor, supersaturated fructose as substrate, and ultrasound to produce the highest ester concentration. Factors, specificallyflow rate, esterification time, and ultrasound power, were optimized. The novelty of this study is the use of fluidized bed reactor combined with an ultrasonicator in the synthesis of ester from oleic acid and supersaturated fructose solution.

METHODS

Materials

The materials used were fructose, 2-phenylpropionaldehyde, methanol, tert-butanol, Tween 20, and Tween 80 purchased from Merck KgaA (Darmstadt, Germany). Oleic acid was purchased from AppliChem, while Lipase of *Candida rugosa* and molecular sieve (3Å; 1.6 mm) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Amberlite IRA 96 free base matrix was supplied by Fluka Analytical.

Lipase Immobilization

A hydrophobic matrix was obtained by modification of the free base form of Amberlite IRA-96 with 2-phenyl propionaldehyde, according to Hilmanto et al. (2016). Lipase immobilization was performed following the procedure described by Hidayat et al. (2016).

Supersaturated Fructose Solution

Supersaturated fructose solution was prepared according to Sang et al. (2008) with modifications of the type of solvent, stirring temperature, and incubation time. About 5.4 g fructose (30 mmoles) was dissolved in 27.7 mL tert-butanol at 65°C and 700 rpm for 12 h. Subsequently, the mixture was incubated for 3 hours at room temperature and centrifuged at 3000 g for 5 min. The supersaturated fructose solution was analyzed for sugar concentration using DNS method (Miller, 1959).

Fructose Oleic Ester Synthesis

Fructose oleic ester synthesis was carried out in a jacketed fluidized bed reactor using the immobilized lipase catalyst, following the method by Hidayat et al. (2016) and Ye et al. (2010), with modification of water bath temperature and speed, as well as temperature and time of stirring the mixture of supersaturated sugar solution and fatty acid. A mixture of 20 mL supersaturated fructose solution and oleic acid was stirred at 400 rpm, 30°C for 30 min. Substrate ratio (fructose: oleic acid) for all reactions was 1:2 (moles/moles).

Fluidized bed reactor system has four main components, interconnected components, allowing

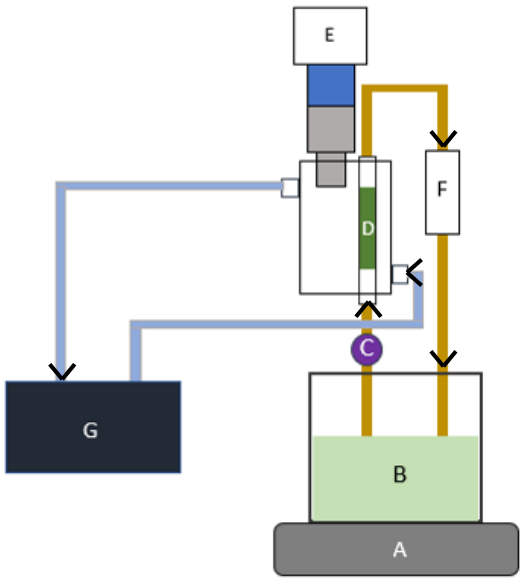

Figure 1. Schematic and design of synthesis of fructose oleic ester (A. hot plate magnetic stirrer, B. reservoir (supersaturated fructose solution mixed with oleic acid), C. peristaltic pump, D. jacketed fluidized bed reactor, E. ultrasound of 20 kHz, F. column of molecular sieve, G. water bath at 50°C).

it to operate as a closed-loop circuit with continuous circulation. The four main components included a reservoir for substrate storage, a peristaltic pump for controlled injection into the fluidized bed reactor, the core jacketed fluidized bed reactor (160 mm x 10 mm, filled with 15% b/b immobilized lipase) equipped with ultrasound (20 kHz), and columns of molecular sieve (110 mm x 10 mm; 12% b/b). Jacketed fluidized bed reactor outer column was filled and recirculated with water from water bath and maintained at 50°C (Abdul Rahman et al., 2012). Figure 1 illustrates the scheme and design of esterification of fructose oleic ester reaction.

Reaction Time on Fructose Oleic Ester Concentration

About 20 mL of fructose supersaturated solution (0.03 moles fructose) was added to a reservoir containing 18.9 mL oleic acid (0.06 moles). The blend was continuously agitated for 30 min at 400 rpm and 30°C under a closed condition. The reaction was initiated by recirculating substrate in the system (Figure 1) at a 0.4 mL/min flow rate. The conditions for the reaction include a temperature of 50°C, with enzyme concentration of 15% (w/w), an ultrasound power of 180 W, and a molecular sieve of 12% (w/w). Sampling was carried out at 0, 10, 20, 30, 45, 60, 90, 120, 180, 240, and 480 min at a place just before the sample drips into the reservoir (Hilmanto et al., 2016).

Ultrasound Power on Fructose Oleic Ester Concentration

About 20 mL of the supersaturated fructose solution (0.03 moles fructose) was added to a reservoir containing 18.9 mL of oleic acid (0.06 moles). The blend was continuously agitated for 30 min at 400 rpm and 30°C under a closed condition. The reaction was initiated by recirculating substrate in the reaction system (Figure 1) at a 0.4 mL/min flow rate. The conditions for the reaction include a temperature of 50°C, with enzyme of 15% (w/w), molecular sieve of 12% (w/w), and various ultrasound powers (0 Watt, 60 Watt, 120 Watt, 180 Watt, and 240 Watt). The reaction was carried out for up to 3 h after using a series of esterification apparatuses (Lee et al., 2008).

Flow Rate on Fructose Oleic Ester Concentration

About 20 mL of fructose supersaturated solution (0.03 moles fructose) was added to a reservoir containing 18.9 mL of oleic acid (0.06 moles). The blend was continuously agitated for 30 min at 400 rpm and 30°C under a closed condition. The reaction was initiated by recirculating substrate in the reaction system (Figure 1). The conditions for the reaction include a temperature of 50°C, enzyme concentration of 15% (w/w), ultrasound power of 180 W, molecular sieve of 12% (w/w), and different flow rates (0.2 mL/min, 0.4 mL/min, and 0.6 mL/min). The reaction was carried out for up to 3 h after using a series of esterification apparatuses (Hidayat et al., 2016).

Assessment of Immobilized Lipase Esterification Activity

Esterification activity was evaluted following method by Hilmanto et al. (2016). The immobilized lipase's activity in the esterification reaction was measured with ethanol and oleic acid serving as substrates. Approximately 0.1 g of the immobilized lipase was added to a reaction mixture containing 0.05 M ethanol in isooctane and 0.05 M oleic acid in isooctane. The mixture was incubated for 20 minutes at 60 °C with shaking at 150 strokes/min. The reaction was quenched by immersing the mixture in an ice bath. The remaining fatty acid content was measured spectrophotometrically at 715 nm. Activity of esterification was reported in units of micromoles (µmoles) of ester produced per minute per gram of immobilized matrix.

Analysis of Fructose Oleic Ester Properties

Analysis of FOE was conducted employing FT-IR (Fourier Transform Infrared) spectroscopy and the TLC-scanning technique reported by Hidayat et al. (2016). The emulsion capacity was analyzed using a modified procedure based on the methodology described by Neta et al. (2012). The emulsion capacity was tested by first blending 3 mL of FOE with 37.5 mL of palm oil and 75 mL of distilled water. The mixture was homogenized at 10,000 rpm for 30 s. An additional 37.5 mL of palm oil was subsequently incorporated, and homogenization was repeated for 90 sec. The resulting emulsion was then centrifuged for 5 min at 3000 g, after which the amount of the emulsion layer was quantified. Stability of emulsion was tested by heating for 30 min in a water bath at 80°C, followed by cooling to room temperature. The sample was re-centrifuged for 5 minutes at 3000 g, and the final volume of the remaining emulsion layer was measured to assess its stability after thermal stress. The size distribution of droplets of the emulsions was analyzed using PSA (Zetasizer Malvern).

RESULTS AND DISCUSSION

Immobilized Lipase Esterification Activity

Activity of esterification of immobilized lipase in the matrix was 36.45 ± 9.95 U/g matrix. It was affected by the adsorbed lipase amount in the matrix. The more lipase

was adsorbed, the greater the esterification activity. Enzyme leakage (lipase) from the matrix may also influence the measured catalytic activity.This was caused by weak adsorption due to the conformational changes (Idris & Bukhari, 2012). The presence of hydrophobic environment in the modified matrix could maintain the adsorbed, preventing easy separation from the matrix, a result of the hydrophobic interaction between the enzyme and the surface of hydrophobic matrix.

Fructose Concentration in Supersaturated Fructose Solution

Fructose concentration of solution of supersaturated fructose was 12.30±2.33 mg/mL, which is relatively low. This can be attributed to the low relative polarity of tert-butanol, in which the relative polarity is 0.389. Considering that fructose is very polar, the solubility of fructose in the tert-butanol is very low. As a comparison, the relative polarity of ethanol is 0.654. A high level of relative polarity resulted in a greater level of polarity. The solubility of another monosaccharide, such as glucose (temperature at 25°C), was also low in tert-butanol, which was 0.3 g/L, while the level ranges from 0.5-145 g/L in ionic liquids (Sang et al., 2008).

Effect of Esterification Time on Fructose Oleic Ester Concentration

Figure 2 shows the effect of esterification time on FOE concentration. FOE increased with a rise in reaction time to 242 min (85.02±1.3%), suggesting that a supersaturated fructose solution also caused a high concentration of FOE. A supersaturated fructose solution could enhance the reaction because fructose in liquid form can be easily transported or diffused into enzyme active site, which allows contact between substrate and active site of enzyme without any obstacles. This condition increases the reaction rate and FOE concentration. Ultrasound also affected enzyme activity by enhancing substrate transfer to the enzyme active site and the product release rate (Lee et al., 2008). Further increase in the reaction time to 302 min and 542 min resulted in a slight decrease in ester concentration, namely 84.01±0.55% and 82.53±4.28%, respectively. Therefore, fructose oleic ester was not significantly different, with an increase in reaction time of 542 min. The best-selected esterification time was 242 min or 180 min.

Ultrasound Power Effect on Fructose Oleic Ester Concentration

Figure 3 illustrates the ultrasound power effect on the ester concentration. Based on Analysis of Variance (ANOVA) analysis, ultrasound power significantly affected ester concentrations at reaction times 92, 182, and 242 min. The highest ester concentration of 85.77±0.69% was obtained at ultrasound power of 180 W for 242 min. Ultrasound power also caused FOE concentration to increase with higher reaction time. Furthermore, ultrasound power of 60 and 120 W produced a higher FOE concentration than that of 240 W. High FOE concentration indicated that the use

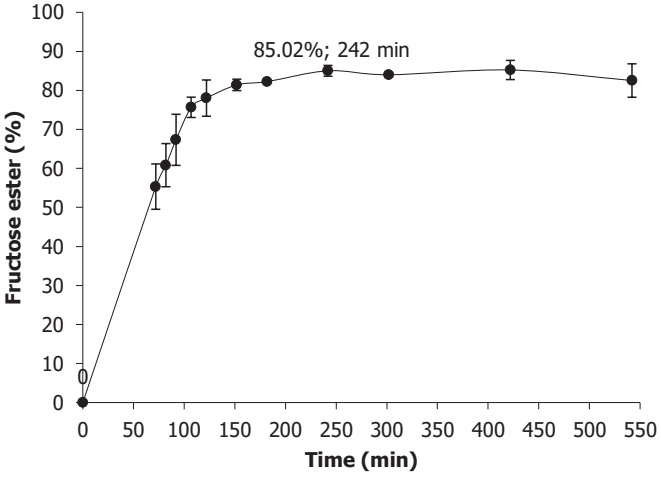

Figure 2. Effect of esterification time on FOE synthesis (ester concentration). Process conditions: temperature at 50°C, flow rate of 0.4 mL/min, ultrasound power of 180 Watt, immobilized lipase of 15% (b/b), molecular sieve of 12% (b/b), and substrate ratio of 1:2.

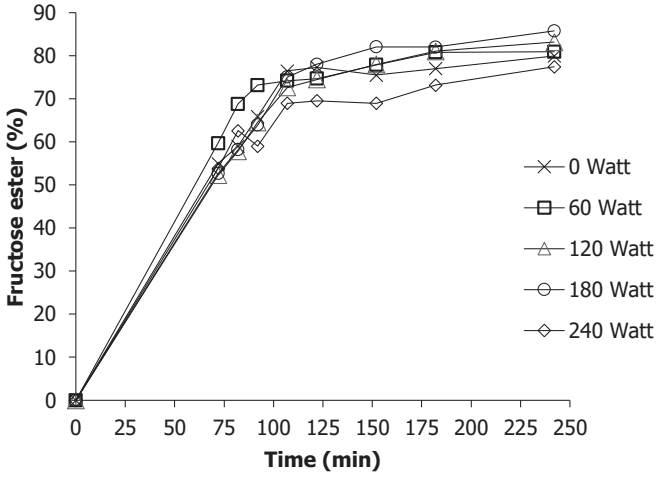

Figure 3. Ultrasound power effect on FOE synthesis (ester concentration). Process conditions: temperature at 50°C, flow rate of 0.4 mL/min, immobilized lipase of 15% (b/b), molecular sieve of 12% (b/b), and substrate ratio of 1:2. Two replications were carried out.

of ultrasound power improved the, accelerating the transfer of substrate and the product release rate (Lee et al., 2008).

In another study, Ishimori (1981) reported that a decrease in ultrasound power accelerated the activities of free and immobilized α-chymotrypsin in aqueous media. Brenelli & Fernandes (2003) reported that ultrasound increased about 3.5 and 10 times mass transfer rates (transfer acyl) than a magnetic stirrer. Ultrasound cavitation open enzyme structure and increase enzyme activity by expanding the surface area and the transfer rate (More et al., 2017).

Ultrasound power of 240 W produced the lowest FOE concentration. This is because higher ultrasound power results in a greater pressure in fluidized bed reactor column and substrate aggregation, which could inhibit contact between substrate and enzyme active site. According to a previous study, higher ultrasound power enhances substrate and enzyme complex release, damages the immobilized lipase, and reduces the immobilized lipase activity. More et al. (2017) added that high ultrasound power could cause inactivation of enzyme.

Substrate Flow Rate Effect on Concentration of Fructose Oleic Ester

Flow rate affects the contact time of substrate with enzyme in the reactor column. Substrate flow rate effect on concentration of FOE is illustrated in Figure 4. The lower flow rate of substrate yielded in a longer reaction time. The breakthrough of substrate was determined after flowing through a reactor system and reentering the reservoir. Based on the results, the breakthrough times at a flow rate of 0.2 mL/min, 0.4 mL/min, and 0.6 mL/min were 124 min, 62 min, and 41 min, respectively.

Ester concentration increased to 84.81±1.50% with a rise in the reaction time to 244 min at the flow rate of 0.2 mL/min. Furthermore, an increase in the reaction time did not significantly affect FOE concentration. For a flow rate of 0.4 mL/min, FOE concentration increased to 76.64% with a rise in the reaction time to 107 min. A slight increase (81.86±2.00%) was obtained with a further rise in the reaction time. An increase in the reaction time did not significantly affect FOE concentration. Finally, for a flow rate of 0.6 mL/min, FOE concentration increased about 1.12 times with an elevation in the reaction time to 71 min. The best concentrations of ester were 85.13±9.56% (304 min), 80.83±2.25% (242 min), and 82.68±2.37% (221 min) for a flow rate of 0.2 mL/min, 0.4 mL/min, and 0.6 mL/min (Figure 4). However, the ANOVA results showed that substrate flow rate did not significantly affect ester concentration for a longer reaction.

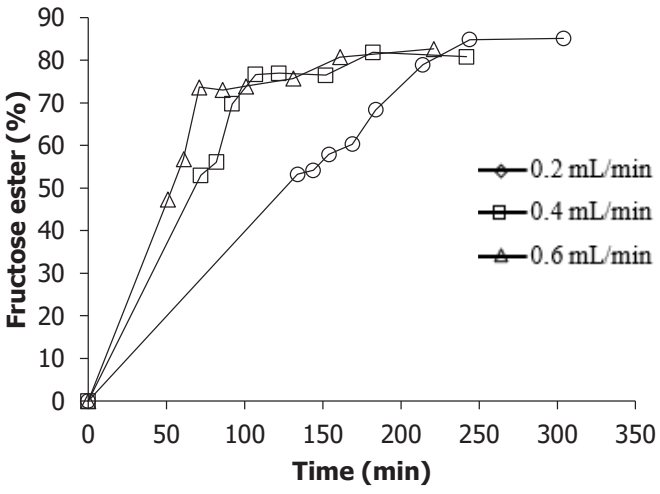

Figure 4. Substrate flow rate effect on FOE synthesis (ester concentration). Process conditions: temperature at 50°C, ultrasound power of 180 Watt, immobilized lipase of 15% (b/b), molecular sieve of 12% (b/b), and substrate ratio of 1:2. Three replications were performed.

FT-IR Analysis of Fructose Oleic Ester

FT-IR analysis showed a peak at wavenumber ~1712 cm^{-1} in all three FOE samples (Figure 5), and

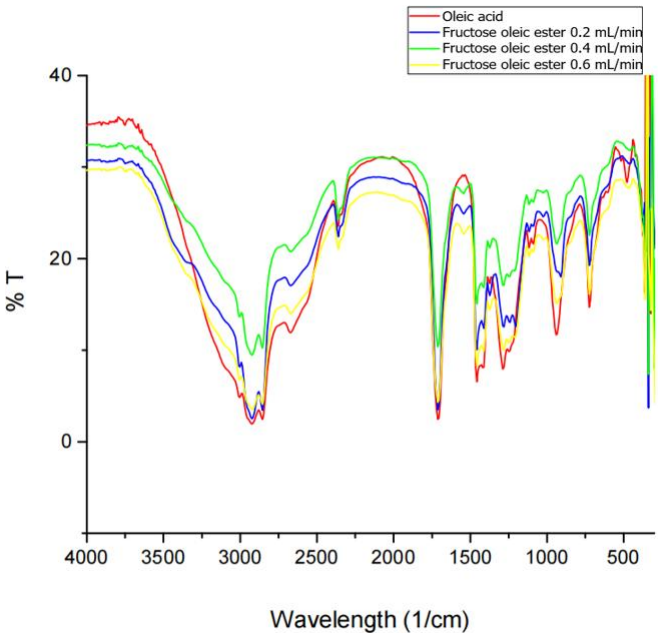

Figure 5. FT-IR spectra of oleic acid; Fructose Oleic Ester at flow rate of 0.2 mL/min; Fructose Oleic Ester at flow rate of 0.4 mL/min; and Fructose Oleic Ester at flow rate of 0.6 mL/min.

Table 1. Emulsion capacity, emulsion stability, average size of droplet, and polydispersity index (PDI) of FOE with commercial emulsifiers

Sample	Emulsion capacity (%)	Emulsion stability (%)	Average size of droplet (µm)	PDI
FOE (0.2 mL/min)	99.86±0.01	99.29±0.04	1.00	1.00
FOE (0.4 mL/min)	99.65±0.00	98.95±0.50	2.00	0.71
FOE (0.6 mL/min)	99.65±0.00	98.59±0.00	5.42	0.33
Control (without *emulsifier*)	91.95±0,00	0±0,00	-	-
Tween 20	99.18±0.67	99.50±0.22	8.03	0.05
Tween 80	99.32±0.02	99.32±0.02	4.42	0.18

oleic acid had a peak at ~ 1712 cm^{-1}. However, the peak area of oleic acid was higher than FOE. This indicates that the absorption band at wavenumber ~1712 cm-$^{-1}$ in the three FOE samples corresponds to the carboxylic functional group. The peak area of carboxylic group in ester product was lower than that of pure oleic acid, suggesting that oleic acid reacted with fructose.

According to Coates (2000), carboxylic group of fatty acid was found in wavenumbers 1725-1700 cm^{-1}. Meanwhile, ester compound was recognized by the existence of a C=O (carbonyl group) bond in its chemical composition. The same group was also found at C=O in oleic acid due to the presence of long chains of hydrocarbons and carboxylic acids (R-COOR). According to Van Den Broek & Boeriu (2013), C=O group (ester) was at wavenumber 1751-1716 cm^{-1}, but this group was also found at 1750-1725 cm^{-1} (Coates, 2000) and 1900-1650 cm^{-1} (Dachriyanus, 2004).

A previous study by Hidayat et al. (2016) found ester group (C=O) (using fructose and oleic acid as substrates) at wavenumber ~1712 cm^{-1}, while Boruczkowska et al. (2012) discovered ester group from potato starch and oleic acid at wavenumber 1715 cm^{-1}. In addition, a new peak at wavenumber 1373 cm^{-1} (C-O bond) was assumed to be a bond or group formed from esterification between fructose and oleic acid. The peak only appeared in FOE samples at 0.2 mL/min.

Emulsion Capacity and Stability

The ability of an emulsifier to form an emulsion can be evaluated from the capacity. In contrast, the ability of emulsifier to maintain after heating is evaluated from the emulsion stability. FOE synthesized from a flow rate of 0.2, 0.4, and 0.6 mL/min, had high emulsion capacity (Table 1). Emulsions were stable after heating and comparable to commercial emulsifiers such as Tween 20 and Tween 80. The control had an emulsion capacity of about 91.95%, but the emulsion was unstable. Therefore, FOE was formed during the synthesis and may be used as emulsifier to stabilize oil in water emulsion.

Droplet Size and Distribution

PSA analysis was carried out to determine emulsion and droplet size distribution. The parameter of particle size distribution in PSA analysis is PDI. The small PDI value indicates that particle size distribution is narrow and uniform. FOE, which was synthesized at 0.2 mL/min, had the smallest droplet size (1.00 µm), but also had the highest PDI value (1.00), suggesting droplets were not uniform. An increase in flow rate to 0.4 and 0.6 mL/min elevated droplet size about 2 and 5.4 times, respectively, and droplet size was more uniform. This suggests that a wide droplet size distribution can be caused by high agglomeration of particles or droplets (Yuan et al., 2008).

The lowest PDI value was obtained from Tween 20, which had lower droplet size distributions along with Tween 80 compared to FOE samples. The small droplet size and uniform droplet size distribution show that the emulsifier forms and maintains good emulsion stability.

CONCLUSIONS

In conclusion, fructose oleic ester was synthesized in fluidized bed reactor using immobilized lipase of *Candida rugosa* in a hydrophobically modified matrix, supersaturated fructose solution, and ultrasound. The best conditions for FOE synthesis were at esterification time, ultrasound power, and substrate flow rate, of 180 min after through one time a series of esterification apparatus, 180 Watt, and 0.2 mL/min, respectively. Concentration of FOE was 85.13±9.56%, yielding a Rf value of 0.2 to ~0.8, wave absorption band for ester group (C=O) at wavenumber ~1712 cm^{-1} with

a new peak (C-O bond) at wavenumber 1373 cm^{-1}, emulsion capacity 99.86±0.01%, emulsion stability of 99.29±0.04%, and droplet size of 1.00 µm with non-uniform distribution of droplet size (PDI=1.00).

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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