Enzymatic Synthesis of Bio-Surfactant Fructose Oleic Ester Using Immobilized Lipase on Modified Hydrophobic Matrix in Fluidized Bed Reactor

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Abstract

Enzymatic synthesis of fructose oleic ester (FOE) in stirred tank reactor using the immobilized lipase may cause a physical damage of support matrix, which causes a leaching of lipase from matrix. The objective of this research was to evaluate the best condition of FOE synthesis in fluidized bed reactor (FBR) using the immobilized lipase on modified hydrophobic matrix. Surface of Amberlite IRA 96 was modified with 2-phenylpropionaldehyde to obtain hydrophobic surface. Candida rugosa lipase was immobilized on the modified matrix. Subsequently, it was used for FOE synthesis in FBR system. Factors, such as reaction time, substrate flow rate, amount of water adsorbent, and substrate molar ratio were evaluated based on the conversion of oleic acid into FOE. Results from FTIR analysis showed that modified matrix had a peak at a specific wavelength ~1674 cm\(^{-1}\). It indicated that a group of imine (-C=N-) occurred after surface modification. The highest adsorption of lipase was obtained after adsorption for 45 min (13.28±0.57 mg lipase/g matrix), which was equivalent to 52.95±1.67%. The lipase activity was 73.66±5.35 U/g matrix. The best FOE synthesis condition was obtained at flow rate of 0.4 mL/min, molecular sieve of 12 %, substrate molar ratio (fructose : oleic acid) 1:4 for 48 h. FOE was confirmed by TLC and FT-IR analysis, which they had \(R_f\) 0.72 and specific wave number of ~1712 cm\(^{-1}\), respectively. FOE had emulsion capacity, emulsion stability, droplet size, and HLB value of 92.83±0.46 %; 85.65±0.92 %; 7.86±0.93 \(\mu\)m; 11-16, respectively. Emulsion type of FOE was oil in water emulsion.

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Keywords: sugar ester, lipase, hydrophobic matrix, fructose-oleic ester, fluidized bed reactor

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1. Introduction

Sugar fatty acid esters (SFAEs) are known as green biodegradable and biocompatible surfactants. SFAEs are non-ionic, non-toxic surfactants, which have high emulsion capacity and stability. They are used in cosmetics, detergents, pharmaceuticals, and also food industries. Esterification, which uses sugar and fatty acid as reactants, depends on type of acyl donor and acyl acceptor. Sugar fatty acid esters derived from fructose has a higher interfacial tension values than other sugar fatty acids based on its acyl and donor acceptor.

SFAEs generally can be produced by chemical (Laksmono et al., 2008; Purwaningtyas & Pramudono, 2009) and enzymatic method (Ha et al., 2010a; Ha et al., 2010b; van Kempen et al., 2013; Ye & Hayes, 2011). Disadvantage chemical method is that the reaction is performed at high temperature, requires high energy and produces browning compound on the product. The use of alkali as a catalyst in the chemical synthesis of esters may be resulted in toxic residues that are difficult to be degraded. Enzymatic synthesis will generate more specific reaction. This is because of the nature of the enzyme specificity. In addition, the enzymatic synthesis is carried out under conditions that do not require high energy and furthermore it produces products that are not toxic compared with chemical processes (Sabeder et al., 2006). Free enzyme is not stable and not re-useable (Chen et al., 2012; Damnjanovic et al., 2012). Enzymes in the immobilized form are more stable, easier to use in continuous system and re-useable compared with the free enzyme.

Esterification process can be performed in a stirred tank reactor (STR), a packed bed reactor (PBR), or a fluidized bed reactor (FBR) (Damnjanovic et al., 2012; Hajar & Vahabzadeh, 2014; Liu et al., 2008; Serri et al., 2010). The use of agitators in the STR has the disadvantage. It can cause stress to the immobilized enzyme and damage on the surface of the support matrix, which causes the leaching of lipase from matrix. Esterification in PBR and FBR may overcome this problem because they may be reduced enzyme stress and damage of the support matrix.

On the other hand sugar as substrate, in the form of crystals, has a large size, which can reduce the substrate flow in the column of PBR. Furthermore, it can cause clogging of column. FBR may overcome this problem. Fluidization of matrix in the column may result in better mixing and matrix distribution in the column, as well as minimizing the pressure on the matrix compared with PBR system (Damnjanovic et al. 2012; Hajar & Vahabzadeh, 2014).

The objective of this research was to evaluate the best condition of FOE synthesis in fluidized bed reactor (FBR) using the immobilized lipase. Lipase was immobilized on the matrix that was modified by the addition of 2-phenylpropanaldehyde. Factors, such as reaction time, substrate molar ratio of fructose: oleic acid and the use of water adsorbent agent were evaluated.

2. Materials and Methods

2.1. Materials

Candida rugosa lipase, molecular sieve 3Å 1.6 mm and 99.99% of olive oil obtained from Sigma-Aldrich (St. Louis, MO, USA). Fructose, 2-phenylpropanaldehyde, Na2SO4-anhydrous were obtained from Merck KGaA (Darmstadt, Germany). Oleic acid 88% was obtained from AppliChem. Amberlite IRA-96 free base was obtained from Fluka Analytical.

2.2. Preparation and modification of matrix for enzyme immobilization

Matrix Amberlite IRA-96 free base was immersed in a buffer solution of pH 12 and incubated in a shaker water bath for 2 h at 30°C. A matrix modification was performed by the addition of 2-phenylpropanaldehyde 12.5% (v / v) in methanol. It was mixed using water bath shaker for 2 h at 30°C. Matrix was then filtered and dried overnight. The modified matrix was characterized using FT-IR analysis.

2.3. Lipase immobilization

The immobilization of lipase into the hydrophobic modified matrix was performed according to Serri, et al. (2010) and Öztrük (2001) with modifications. Lipase (powder) was dissolved in phosphate buffer pH 7 containing 1
M NaCl (5 mg / mL). Mixture was then stirred at cold temperature for 15 min and then centrifuged at 2000 rpm for 15 min. Furthermore matrix was equilibrated in pH 7 phosphate buffer containing 1 M NaCl and further it was separated prior to the addition into solution of lipase (5 mg / mL). Mixture was incubated in a shaker water bath for 1-120 min at 37°C and 150 rpm. Matrix was then filtered and kept at 4°C. The best adsorption time was determined according to the amount of adsorbed protein and lipase hydrolytic activity. The amount of adsorbed lipase was assumed as the adsorbed protein into the matrix. Lipase loading was defined as the amount of protein that adsorbed into the support matrix (mg lipase/g matrix), calculated as the difference between the amount of the added protein (mg) during the immobilization process and the amount of protein (mg), which was found in solution and washing buffer after the immobilization.

2.4. Assay of hydrolytic activity

Lipase hydrolytic activity assay was performed according to (Marseno et al., 1988) with some modifications. Hydrolytic reaction was initiated by adding the immobilized lipase into a flask containing 5 ml of olive oil in isooctane (60 : 100 v/v). The mixture was incubated at 37 °C for 20 min. The reactions were stopped by placing the flasks in an ice bath.

The forming of free fatty acid was further determined as follow. Sample was dissolved in 1.8 ml isooctane. About 0.4 ml cupric acetate pyridine was further added. The mixture was mixed for 10 min, and then the absorbance of the supernatant phase was measured by spectrophotometer at 715 nm. Unit activity was expressed as the amount of the hydrolyzed free fatty acid (µmol) per min per gram matrix.

2.5. Synthesis of fructose oleic ester

The synthesis of oleic fructose ester was performed in a FBR using lipase, which was immobilized on the modified hydrophobic matrix (Fig. 1). FBR had three main components, which were interconnected with each other to form a closed-loop system. The three main components were including a 50 mL erlenmeyer in an open condition as a reservoir, peristaltic pumps for pumping the substrate into the reactor, and jacketed fluidized bed reactor (160 × 10 mm, filled with 2 g of the immobilized lipase; 147.3 U). Reservoir was set at 60°C and stirred at a speed of 400 rpm using a hot plate magnetic stirrer. The jacketed column was filled with re-circulated water and maintained at 60°C (Sabeder et al., 2006).

Conversion of oleic acid into FOE (%) was used as an indicator to evaluate the synthesis of fructose oleic esters in the fluidized bed reactor using the immobilized lipase on modified matrix. Conversion (%) was defined as the amount of oleic acid that reacted with fructose divided by the initial amount of oleic acid times 100%.

Fig. 1. Synthesis fructose oleic ester in fluidized bed reactor (FBR). FBR was containing the jacked column (160 x 10 mm) and the immobilized lipase (A), peristaltic pump (B), substrate reservoir (C) and hot plate stirred (D).
2.6. Characterization of fructose oleic ester

FOE was characterized by TLC-scan (Dandekar & Patravale, 2009) and FT-IR methods. Analysis of the emulsion capacity was performed according to Neta et al. (2012) with modification. About 3 mL FOE was added into 37.5 mL of palm oil and 75 mL of distilled water. The mixture was homogenized for 30 sec at 10,000 rpm. Furthermore 37.5 mL of oil was then added again and homogenized for 90 sec. The mixture was further centrifuged at 3,000 g for 5 min. The amount of emulsion layer was then calculated.

Emulsion stability was determined by heating the emulsion at 80°C for 30 min in a water bath, and subsequently it was cooled to room temperature. Subsequently, it was centrifuged at 3,000 g for 5 min, and the volume of the emulsion layer was measured.

3. Results and Discussion

3.1. Characterization of modified matrix

Amberlite IRA-96 is an anion exchange matrix. Soaking of Amberlite IRA-96 in a buffer solution pH 12 was performed to increase the amount of free primary amine. The results from FT-IR analysis showed that the characteristic absorption band of the native matrix occurred on the wave number range from 3400 cm\(^{-1}\) and 2927 cm\(^{-1}\) due to the presence of an amine functional group (NH\(_2\)). Peaks at wave number 1604 cm\(^{-1}\) and 1456 cm\(^{-1}\) indicated the vibration of the primary NH and the stretching vibration of C=C derived from aromatic ring, respectively. Coates (2000) reported that the characteristic of the amine groups was associated with the location of molecular nitrogen. It could be divided into several parts, namely, primary amines, secondary amines, tertiary amines, and aromatic amines. Wave number of 3400-3380 cm\(^{-1}\) was characteristic for a primary amine with NH stretching vibration and wave number 1650-1590 cm\(^{-1}\) was characteristic for a secondary amine with NH undergo bending vibration, while the wave number in the range 1510 to 1450 cm\(^{-1}\) indicated the bending vibration of the aromatic ring C=C. Typical absorption band of 2-phenylpropionaldehyde was at wave number 1800-1700 cm\(^{-1}\). Damnjanovic et al. (2012) also found the same characteristic absorption band for the amine group at wave number ~ 3400 cm\(^{-1}\) and 2934.52 cm\(^{-1}\).

Modification of matrix resulted in the formation of a covalent bond between the amine group (-NH\(_2\)) and the aldehyde group of 2-phenylpropionaldehyde. The results of FT-IR spectra showed the occurring of a new bond at wave number ~1674 cm\(^{-1}\), which was the vibration of the imine group -C=N- bond. This peak indicated that the reaction between the amine group (-NH\(_2\)) of the native matrix and 2-phenylpropionaldehyde to form imine functional groups (-C=N-). Imine functional groups could be determined based on the characteristic of the wave number 1690-1590 cm\(^{-1}\). It indicated the modification of matrix with 2-phenylpropionaldehyde was successfully carried out. Thus matrix became hydrophobic due to the attachment of 2-phenylpropionaldehyde on the matrix surface.

3.2. Lipase immobilization

Fig. 2 shows the effect of the adsorption time on the adsorbed lipase on the matrix. The amount of the adsorbed lipase increased from 11.34 ± 2.02 mg/g to 13.22 ± 0.41 mg/g matrix, when the adsorption time increased from 1 min to 45 min. Further increase in adsorption time from 45 min to 120 min did not cause an increase in the amount of the adsorbed lipase significantly. It is suggested that the immobilization of lipase reached equilibrium after 45 min. A similar trend was also reported by Öztrük (2001). The immobilized lipase on octyl sepharose reached 49% after 20 sec adsorption and it reached 94% after 5 min adsorption. Öztrük (2001) also reported that the level of hydrophobicity of the support matrix effect the immobilization of lipase. The more hydrophobic a matrix will be more and more lipase adsorbed into the matrix. It is also known that the time to reach equilibrium is shorter. Hydrolytic activity of the immobilized lipase increased from 39.37 U/g matrix to 73.66 U/g matrix, when the adsorption time increased from 1 to 45 min. An increase in hydrolytic activity related to the increase in the number of the adsorbed lipase on the matrix. The highest activity was achieved after 45 min. Further increase in the adsorption time resulted in no significant increase in lipase activity. Selection of the best adsorption time was based on...
on a high adsorbed lipase enzyme activity. Therefore the adsorption time of 45 min was chosen as the best adsorption condition. The immobilized lipase on this modified matrix was further used to synthesize esters of oleic fructose in the fluidized bed reactor.

![Figure 2](image)

**Figure 2.** Effect of adsorption time on both the immobilized lipase and its hydrolytic activity. Adsorption was performed at 37°C, pH 7 containing of 1 M NaCl.

### 3.3. Effects of reaction time on synthesis of fructose oleic ester

The reaction time is converted in term of cycle number. One cycle is defined as the required time of the fluid flows from the reservoir into the FBR column and back to the reservoir. Fig. 3 shows the effect of the reaction time on the ester conversion. The results showed that an increase in the conversion of substrate into FOE occurred and reached 80.32±11.97% at 67.32 cycles or for 48 hr. Meanwhile, conversion decreased about 25.04% at 100.98 cycles or reaction time of 72 hr. It is suggested that a decrease in conversion is due to production of water during the esterification process, which initiates the hydrolytic reaction. The addition of water adsorbent agent may reduce the amount of water in the system and improves substrate conversion.

![Figure 3](image)

**Fig. 3.** Effect of incubation time on the synthesis of fructose oleic ester. Reaction was performed at 60°C, substrate flow rate of 0.5 mL/min (42.78 min per cycle), Na₂SO₄·Anhydrous 12% as water adsorbent, and substrate ratio of fructose: oleic 1: 4. %. FOE was calculated as fructose mono-oleic fructose ester.
The obtained FOE may be a mixture of mono-, di-, and tri-esters. *Candida rugosa* lipase is a non-specific lipase, it catalyst the reaction between oleic acid and hydroxyl groups of substrate at random, especially when there is a primary -OH group then followed by secondary -OH group. Fructose has 5 hydroxyl groups, which are two primary hydroxyl groups and two secondary hydroxyl groups, so that it allows the binding of more than one oleic acid on fructose molecules. From these results, the best substrate conversion was found at 67.32 cycles, in which yield was 80.32±11.97%. For the next stage, the esterification time of 48 hr was used for the esterification reaction in the FBR system.

3.4. Effects of substrate flow rate on substrate conversion

Effect of substrate flow rate can be seen in Fig. 4. The flow rate relationship, a long time of one cycle and the conversion yield of ester can be seen in Table 1. After 48 hr of the esterification reaction the highest substrate conversion was obtained at a flow rate of 0.1 mL/min, in which substrate conversion based on oleic acid was 197.06±14.16% monoester. The lowest conversion was at a flow rate of 0.5 mL/min, in which the conversion was 86.32±11.41% monoester. Substrate conversion exceeded 100% indicated that a mixture of mono-, di-, tri-esters was formed in the final product. This phenomenon may occur due to the excessive length of reaction time.

![Fig. 4. Effect of substrate flow rate on the synthesis of fructose oleic ester. Reaction was performed at 60°C, Na₂SO₄-Anhydrous 12% as water adsorbent, substrate molar ratio of fructose: oleic 1: 4, for 48 hr. FOE was calculated as fructose mono-oleic fructose ester.](image-url)

Theoretical calculation of FOE based on mono-oleic fructose ester was 87.42 ± 11.52% at a flow rate 0.4 mL/min for 48 hr reaction (Table 1). However FOE was a mixture of mono-, di-, and tri-oleic fructose esters at a lower flow rate. It is suggested that mono-oleic fructose ester was further reacted with other oleic acid by the enzyme to form di- or tri-oleic fructose ester at a lower reaction time due to both the availability of high amounts of oleic acid and the presence of secondary hydroxyl groups (Serri et al., 2010). An increase in flow rate to 0.5 mL/min did not significant increase in the conversion of substrate. Thus it was found that the best FOE synthesis for producing mono-oleic fructose ester was performed at a flow rate of 0.4 mL/min. When the reaction was performed for 48 hr, the lower substrate flow rate resulted in the conversion of mono-oleic fructose ester into di- and tri-oleic fructose esters.
Table 1. Effects of substrate flow rate on the forming of the calculated mono-, di- and tri-oleic fructose ester

<table>
<thead>
<tr>
<th>Flow Rate (mL/min)</th>
<th>Time per cycle (min)</th>
<th>Total cycles*</th>
<th>% esterification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mono-ester</td>
</tr>
<tr>
<td>0.1</td>
<td>213.91</td>
<td>13.46</td>
<td>197.06 ± 14.16</td>
</tr>
<tr>
<td>0.2</td>
<td>106.96</td>
<td>26.93</td>
<td>152.74 ± 11.61</td>
</tr>
<tr>
<td>0.3</td>
<td>71.30</td>
<td>40.39</td>
<td>123.57 ± 14.87</td>
</tr>
<tr>
<td>0.4</td>
<td>53.48</td>
<td>53.85</td>
<td>87.42 ± 11.52</td>
</tr>
<tr>
<td>0.5</td>
<td>42.78</td>
<td>67.32</td>
<td>86.32 ± 11.41</td>
</tr>
</tbody>
</table>

* Reaction time was converted into reaction cycles. Reaction was performed for 48 hr

3.5. Effects of water adsorbent agent

The effect of the water adsorbent in the synthesis of FOE in FBR can be seen in Fig. 5. It shows that the addition of Na2SO4 anhydrous and molecular sieve as water adsorbent resulted in an increase in the conversion of substrate into FOE about 1.61 and 1.68 times, respectively. There was not significant different between the addition of Na2SO4 anhydrous and molecular sieve. It is suggested that the esterification reaction produced FOE and also water as products. On the other hand esterification reaction was favorable when water in the reaction system was limited (van den Broek & Boeriu, 2013). As a consequence, producing water during reaction resulted in a changing of the esterification reaction equilibrium into a favorable hydrolytic reaction. The addition of water adsorbent agent reduced the amount of water in the reaction system and therefore it increased the substrate conversion. However, molecular sieve is more easier to be separated. Therefore molecular sieve (12%) was chosen for further experiments.

Fig. 5. The effect of the water adsorbent agent addition on the synthesis of fructose oleic ester. Reaction was performed at 60°C, substrate flow rate of 0.4 mL/min (53.48 min per cycle), substrate molar ratio (fructose: oleic) of 1: 4, for 48 hr. FOE was calculated as fructose mono-oleic fructose ester.
3.6. Effects of molar substrate ratio

The effect of the molar substrate ratio (fructose: oleic acid) on the synthesis of FOE can be seen in Fig. 6. The highest substrate conversion was obtained at a substrate molar ratio of 1:6. The conversions of substrate into FOE were 207.69±30.48%, 103.85±15.24% and 69.23±10.16% based on the calculated mono-, di- and tri-oleic fructose ester, respectively, when the reaction was 48 hr or 53.48 cycles. Based on this calculation it is suggested that FOE was a mixture of mono-, di-, and tri-esters. On the other hand, the conversions of substrate into FOE based on the calculated mono-oleic fructose ester at the substrate molar ratio of 1:4 and 1:2 were 91.69±7.11% and 87.80±11.56%, respectively. An increase in sugar molar ratio resulted in a favorable the forming of monoester, while sugar concentration decreased resulted in more favorable the forming of di-ester and tri-ester (Dubreucq et al., 2000). Thus di- and tri-ester were dominant at the substrate molar ratio of 1:6. The best substrate molar ratio on the synthesis of FOE was 1:2 and 1:4 (fructose: oleic acid), in which mono-ester was dominant.

![Fig. 6. Effect of substrate molar ratio on the synthesis of fructose oleic ester. Reaction was performed at 60°C, substrate flow rate of 0.4 mL/min (53.48 min per cycle), molecular sieve 12% as water adsorbent, for 48 hr. FOE was calculated as fructose mono-, di and tri-oleic fructose ester.](image)

3.7. Characterization of fructose oleic ester

Synthesis FOE at the substrate molar ratio of (fructose: oleic acid) 1:2, 1:4, and 1:6 produced a mixture of mono-, di-, and tri-ester. Identification FOE using TLC Scanner resulted Rf values between 0.72 and 0.76. From FT-IR spectra analysis the ester group of FOE products was detected according to the appearance of a typical absorption band at wave number 1712 cm\(^{-1}\). Ester compounds were identified by the presence of the C=O bond in the molecule. Some studies showed a similar absorption pattern. Typical wave number for the ester (C=O) were in the ranges of 1716-1751 cm\(^{-1}\) (Coates, 2000; van den Broek & Boeriu, 2013). This was also confirmed by Boruczkowska et al. (2012), Zaidan et al. (2012) & Syamsul et al., (2010) that the wave number of the ester were 1715 cm\(^{-1}\), 1723 cm\(^{-1}\), and 1732 cm\(^{-1}\), respectively.

An important parameter for evaluating the power of an emulsifier is the emulsion capacity and stability. Capacity, stability, and droplet size of the emulsion can be seen in Table 2. FOE from molar substrate ratio of 1:2 and 1:4 had a high emulsion capacity. The emulsion capacity of both FOE were not significant different. However, the emulsion stability was significantly different. Droplet size of FOE from substrate molar ratio 1:2 was 1.67 times smaller than from substrate molar ratio 1:2. It is suggested that FOE from substrate molar ratio 1:2 had more mono-oleic fructose ester than from substrate molar ratio 1:4. Therefore FOE from substrate molar ratio 1:2 had characteristic better than from substrate molar ratio 1:4. Emulsion type of both FOE was oil in water emulsion.
Table 2. Emulsification character of Fructose Oleic Ester

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ratio 1:2</th>
<th>Ratio 1:4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsion capacity (%)</td>
<td>92.83±0.46</td>
<td>91.67±2.36</td>
</tr>
<tr>
<td>Emulsion stability (%)</td>
<td>85.65±0.92</td>
<td>81.78±2.52</td>
</tr>
<tr>
<td>Droplet size (µm)</td>
<td>7.86±0.93</td>
<td>13.07±0.93</td>
</tr>
<tr>
<td>HLB value (theory)</td>
<td>11-16</td>
<td>11-16</td>
</tr>
<tr>
<td>Emulsion type</td>
<td>o/w</td>
<td>o/w</td>
</tr>
</tbody>
</table>

4. Conclusions

Modified matrix had a peak at a specific wavelength ~1674 cm⁻¹ indicating the occurring of imine group of (-C=N-) after surface modification. The highest adsorption of lipase was obtained after adsorption for 45 min (13.28 ± 0.57 mg lipase/g matrix). The lipase activity was 73.66 ± 5.35 U/g matrixes. The best FOE synthesis condition was obtained at flow rate of 0.4 mL/min, molecular sieve of 12 %, substrate molar ratio (fructose : oleic acid) 1:4 for 48 h. FOE had F r y 0.72 and specific wave number of ~1712 cm⁻¹. Emulsion capacity, emulsion stability, droplet size, and HLB value of FOE were 92.83 ± 0.46 %; 85.65 ± 0.92 %; 7.86 ± 0.93 µm; 11-16, respectively. Emulsion type of FOE was oil in water emulsion.

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