# Use of Purified Glycerol from Biodiesel Plants for Acylglycerol Production and Use of Acylglycerol in Foods

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## Abstract

**Background and Objective:** Biodiesel is a well-known liquid fuel. However, a large quantity of glycerol is produced as a byproduct during the biodiesel production. If this is used as a substrate for value-added products such as monoacylglycerols, economic viability of the biodiesel process may improve. There are various uses of monoacylglycerol. However, its use as a substrate for oleogel is still a challenge. Therefore, the aim of this study was to assess the optimum acylglycerol production by immobilized lipase using glycerol from biodiesel processing and various plant oils as substrates. Moreover, use of acylglycerol for oleogel production was studied.

**Material and Methods:** First, glycerol was collected from biodiesel plants and purified using repeated cycles of acidification. Purified glycerol and various types of plant oils, including coconut, rice bran and palm oils, were used to produce acylglycerol via immobilized lipase catalysis. Then, acylglycerols from each plant oil were selected and used as substrates for oleogels. Acylglycerol was characterized following standard methods using gas chromatography-mass spectroscopy and Fourier transformed infrared spectroscopy. Moreover, structure and develop of oleogel were assessed using Fourier transformed infrared spectroscopy and scanning electron microscopy.

**Results and Conclusion:** The highest acylglycerol yield (91.15%) was achieved under conditions, including 20% enzyme loading, 6:1 glycerol to palm oil ratio, 8% water content, 40 °C reaction temperature and 24-h reaction time. Acylglycerol from glycerol with palm oil included mono, di and triacylglycerols with contents of 85.0, 10.0 and 2.0%, respectively. Results showed that types of oil included no effects on oleogel qualifications in this study and all oleogels included good characteristics use in foods.

**Conflict of interest:** The authors declare no conflict of interest.

## 1. Introduction

Fats are commonly used in foods for various purposes. However, there are concerns of saturated and *trans*-fatty acids (FAs), which are produced during the manufacturing. Therefore, alternative liquid oils such as oleogel, have been produced to decrease these harmful effects. Structures of the selected oil media for oleogel production and development production processes are important parameters. Currently, oleogels have been interested as alternatives to saturated and *trans*-fatty acids. Oleogels are used in various methods such as nutraceutical delivery, flavors and drugs [1-3]. Oleogels are produced from oleogelator molecules and a large quantity of entrap organic liquid [4]. Oleogels include various in

gelation mechanisms since they can be produced from a variety of structuring agents. Good oleogels should include good stabilities and offer additional advantages for health and environment [5]. Oleogels can be produced from several low-molecular-weight organogelators (LMOGs), including favorable acylglycerol [6, 7]. In this study, acylglycerol was produced from glycerol (a by-product from biodiesel processing) and vegetable oils as substrates for oleogels due to their availability. Biodiesel is a widely accepted liquid alternative fuel that can be produced from various fats, oils and waste grease feedstock [8]. However, a large quantity of glycerol is produced during biodiesel processing. Therefore, use of biodiesel glycerol is still a big challenge. It has been reported that glycerol can be used as a substrate for MAG; hence, economic viability of the biodiesel processing may be improved. However, only a few reports on MAG production from biodiesel glycerol are available. Structure of MAG consists of an aliphatic lipophilic chain and two hydroxyl groups (OH) in the hydrophilic part. Therefore, MAG can be used as an emulsifier, surfactant and stabilize emulsion [9]. In addition, MAG is appropriate to use as a lubricant in textile and fibre industries [10,11]. In fact, MAG and its derivatives represent 75% of the global production of food emulsifiers [12]. The most common method for the production of MAG includes glycerolysis reaction, which is the transesterification of triglycerides with glycerol to MAG [13]. Moreover, MAG can be produced via various reactions, including glycerolysis of alkyl esters, glycerolysis reaction of triglycerides, transesterification of oils with short-chain alcohols and esterification of FAs with glycerol. MAG is commonly producing under high temperatures (225 °C) and only 40-60% of MAG is achieved. Moreover, increased temperature may affect physiochemical characteristics of the final products and their uses [14]. Therefore, alternative methods to overcome the MAG production are important. The MAG production using enzymatic process are possible. However, cost of the enzyme is the most problem. Therefore, immobilization technique is used to progress enzymatic processes for industrial uses [15]. There are various uses of MAGs; they can be used as emulsifiers in cosmetics, pharmaceutical products and foods [16]. However, uses of MAG as a substrate for oleogels is still a challenge for the food industries [17]. Therefore, the aim of this study was to assess the optimum acylglycerol production through immobilized lipase method using glycerol and plant (coconut, rice bran and palm) oils as substrates. To produce acylglycerol lipases were used as catalyzers for commercial and industrial uses. Use of acylglycerol for production of oleogel was studied as well. Indeed, production and optimization conditions of acylglycerol from lipase immobilized on polyhydroxybutyrate (PHB) using purified glycerol from biodiesel plants have never been reported.

## 2. Materials and Methods

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### 2.1. Chemicals and reagents

Lipase powder of *Candia rugosa* and PHB were purchased from Sigma-Aldrich, USA. All other chemicals included analytical grades and purchased from Merck, Germany. For the preparation of immobilized enzymes, *C. rugosa* lipase was immobilized on PHB beads at an activity of 1.94 mg g$^{-1}$ support as described by Binhayeeding et al. [18].

### 2.2. Pretreating glycerol

Biodiesel glycerol was provided by Yala Biodiesel Community Enterprise Group (Yala, Thailand). Crude glycerol was purified based on a method described by Chandra et al. [19]. Crude glycerol (1 kg) and 1.19 M sulfuric acid ($H_2SO_4$) were mixed and pH was adjusted to 2.0. Sample was set until divided into three layers. The middle layer was collected and filtered. Then, the harvested layer was neutralized with 12.5 M NaOH and evaporated at 105 °C for 2 h. The neutralized sample as an enriched glycerol was filtrated and extracted with excess ethanol for 10 min. Then, the glycerol-ethanol solution was collected, filtrated and evaporated at 80 °C for 20 min. After evaporation, pure glycerol was used as the substrate for acylglycerol [20].

### 2.3. Plant oils

Three types of plant oils were used in this study. Palm oil (PO), Gaysorn brand, was purchased from Patum Vegetable Oil, Pathum Thani, Thailand. Rice bran oil (RBO), King Rice Bran Oil brand, was purchased from Thai edible oil, Bangkok, Thailand. Coconut oil (CO), Maprao Thai brand, was purchased from Thai Pure Coconut, Samut Sakhon, Thailand. The three oils were stored at 4 °C until further analysis. The oils were selected because coconut, rice bran and palm oil were the abundant oils in Thailand. Moreover, their characteristics varied [21].

### 2.4. Production of acylglycerols and optimization

Optimization was carried out under batch experiment in 50-mL screw-capped flasks. All parameters and values were selected following previously studies [18]. Purified glycerol (15 g) was mixed with the plant oil (PO, RBO and CO) (20 g) and immobilized lipase (20%), whereas, the other viable were fixed at constant values [22]. Water content was fixed at 5%. Glycerol was mixed with the plant oil at a molar ratio of 6:1 in methanol (25 wt % of the original oil) at 40 °C for 24 h at 250 rpm. Then, organic solvent (25 wt % of the original oil), water content (0-10%) and glycerol mixed with palm oil at a molar ratio of 2:1 to 10:1, enzyme loading (5-30%wt) and incubation temperature (40–60 °C) varied. After each experiment, acylglycerol was collected by centrifugation (4000× g, 20 min). Sample was separated using separating funnel for 12–24 h. The upper layer, the

acylglycerol layer, was collected and washed twice with 20 mL hot deionized water. Then, sample was dried using oven (105 °C) for 24 h. Additionally, CO and RBO were used for acylglycerol production under conditions similar to those used for PO. The acylglycerols from PO, RBO and CO were further characterized and used as substrates for oleogels. Acylglycerol was analyzed using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) and gas chromatography-mass spectroscopy (GC-MS) and acylglycerol production was characterized for emulsion stability and viscosity.

## 2.5. Characterization of acylglycerols

### 2.5.1. Gas chromatography-mass spectrometry

The acylglycerol contents (mono, di and triacylglycerol) were quantified using GC-MS (7890B/5977 Series, Model Agilent Technologies, USA) equipped with a capillary column (HP-5MS, 30 m × 0.25 mm, 0.25 μm thickness) and a mass spectrometer [MSD 5973 (EI)]. The chromatographic conditions included temperature of the MS quad of 150 °C and temperature of the MS source of 230 °C. Helium was used as the carrier gas at a flow rate of 3.0 ml min$^{-1}$ with an injector temperature of 290 °C. Separation conditions included initial temperature of 50 °C then increased to 240 °C at 10 °C min$^{-1}$. The final temperature was constant for 15 min. The GC spectra, including peak percentages and areas, were assessed using integration software. All GC experiments were carried out in triplicate and the values were averaged. Contents of MAG, DAG and TAG were calculated and presented in percentage in acylglycerols and the yield of acylglycerol was calculated as Eq. 1 [23]:

$$\text{Yield (\%)} = 100 - \frac{\text{Resider TAG}}{\text{Total acylglycerol}} \times 100 \qquad \text{Eq. 1}$$

### 2.5.2. Fourier transform infrared spectroscopy

The ATR-FTIR was used to assess the chemical structures of MAG, DAG and TAG with a frequency range of 4,000–400 cm$^{-1}$ [24].

### 2.5.3. Emulsion stability

Standardized method was used to prepare emulsion using glass thermostat connected to a mechanical stirrer (1500 rpm) and a glass paddle agitator. Then, paraffin oil (d20 = 0.8667 g cm$^{-3}$, η20 = 161.8 mPas) and water phases were heated to 800 °C. Mixture was added concurrently to the equipment. The oil to water ratios varied at 20:80, 30:70, 40:60; 50:50, 60:40, 70:30 and 80:20. Emulsifier was dissolved in the oil phase [25]. Phase inversion temperature and volume of the oil phase were continuously measured of temperature and conductivity of the system [26]. Furthermore, the oil phase was collected and assessed for the emulsion stability. The emulsion stability was assessed following a method described by Colucci et al. [25].

### 2.5.4. Viscosity

Viscosity was analyzed using rheometer (Physica Model MCR51, Austria) and probe rotation at a speed of 100 r/min for 180 s, controlling the sample temperature at 25 °C. Viscosity of the sample was read five times [27].

## 2.6. Use of acylglycerols for oleogel production

Oleogels were produced using CO, PO and RBO to form coconut oil oleogel, palm oil oleogel and rice bran oil oleogel. Production of olegel were prepared in screw-capped tubes. First, plant oil was mixed with acylglycerol (7 wt %) under magnetic stirring at 90 °C for 15 min. Then, each sample was cooled down to 25 °C and stored at 25 °C for 48 h before analysis [22]. Sufficient quantities of structuring agent and oil ration were calculated based on the preliminary tilting test to assess the appropriate quantity of oleogelator to produce oleogels. Sample was subjected to scanning electron microscope (SEM) and ART-FTIR for better characterize-ation. Oleogel samples were stored at room temperature (25 °C) for 24 h before characterization using SEM. Then, ethanol was used to remove oils from the sample surface. Gels were washed with ethanol for 3 h. The de-oiled samples were dried at 25 °C for 48 h using vacuum drying chamber. Then, samples were frozen in liquid nitrogen (-210 °C). Images were recorded using SEM (TM3000, Hitachi, Japan) at 15 kV [28].

## 2.7. Statistical analysis

All experiments were carried out in triplicate and data were shown as mean ±SD (standard deviation). All calculations were carried out at 5% significant level. A completely randomized design was used for the analyses. Data were analyzed using analysis of variance. Duncan's multiple range test was used for the mean comparisons. Furthermore, SPSS Software v.11.5 (SPSS, USA) was used for statistical analysis in this study.

## 3. Results and Discussion

### 3.1. Optimization of acylglycerol production

Glycerol wastes from biodiesel and plant oils (including CO, RBO and PO) were used as substrates for acylglycerol. Biodiesel-glycerol generally include a mixture of glycerol, organic materials, inorganic materials, water, soap, salts, alcohols, vegetable colors and trace of glycerides. Therefore, biodiesel wastes were firstly purified before use to improve the quality of glycerol [29]. The biodiesel-glycerol was a liquid with dark color and alkaline pH (8.58 ±0.25). For the purification of glycerol, H$_2$SO$_4$ was added to the solution. After pH reached pH 2, solution was automatically separated into three phases. A middle phase (glycerol-rich) was collected and neutralized with NaOH and extracted with EtOH. Then, purified glycerol with a neutral pH (5.51 ±0.20) was achieved.

### 3.1.1. Effects of substrates

First, acylglycerol was produced by changing the type of substrates, which included PO, RBO and CO. The highest acylglycerol content (74.12%) after 24 h was achieved when palm oil was used (Fig. 1). Pinyaphong et al. [30] reported that coconut oil could produce a mole ratio (8:1) of acylglycerol (58.5%) in ethanol at 45 °C within 36 h. Lu et al. [31] used rice bran oil as substrate for acylglycerol production (39.55%) at 65 °C. However, in the current study, the acylglycerol content was low when using CO (65.23%) and RBO (67.81%) with glycerol; as shown by the previous studies [32-34]; in which, palm oil could produce a high quantity of acylglycerol (68–80%). Satriana et al. [35] reported that the higher MAG content was achieved when a sufficient volume of glycerol reacted with fat/TAG. Therefore, palm oil was selected as the substrate for acylglycerol production in the following experiment.
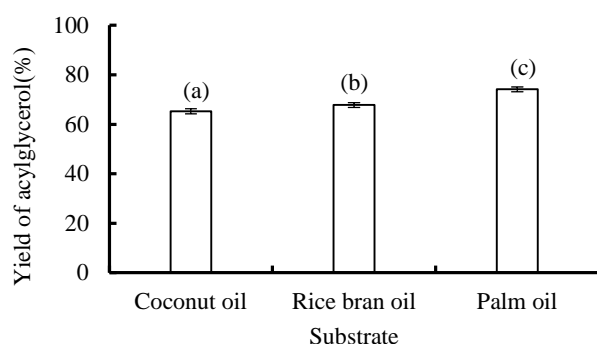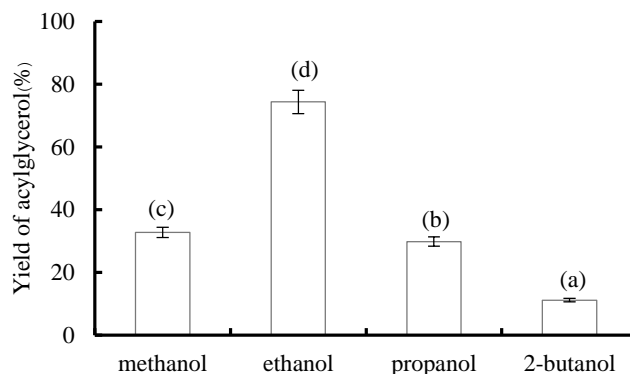


**Figure 1** Effect of substrate on acylglycerol production by immobilized lipase. The process was performed at a 5% water content, and glycerol was mixed with plant oil including CO, RBO, and PO at a molar ratio of 6:1 at 40°C and 250 rpm for 24 h. Bars represent the standard deviation from triplicate determinations. Different letters indicate a statistically significant difference in the means ($p < 0.05$)

### 3.1.2. Effects of organic solvents

Methanol, ethanol, propanol and 2-butanol were selected and used as organic solvents in this study. Organic solvents were generally added into the reaction mixture for improving homogeneity and stability of the system and to decrease viscosity and mass transfer limitations. Moreover, presence of organic media resulted in a better emulsification of glycerol and oil, with an increased surface area accessible for the enzyme. It was possible that this polarity change prevented adherence of glycerol to the enzyme, improving the contact between the oil and the enzyme [36]. The highest acylglycerol content (74.4%; 24 h) was achieved when ethanol was used (Fig. 2). Hydrophilic solvents (such as isopropanol and ethanol) yielded higher MAG contents than those the hydrophobic solvents (such as n-heptane, n-hexane and isooctane) did. Use of ethanol as an organic solvent could improve poor solubility of the substrate in glycerol. High content of MAG in the ethanol system was supported by the findings of Yesiloglu [37]; the optimal yield (81%)

was achieved using sunflower oil and oil/ethanol at a molar ratio of 1:3. Previously, Irimescu et al. [38] reported that the highest 2-MAG was achieved when a larger volume of ethanol was used. The optimal conditions for 2-MAG included an ethanol to oil molar ratio of 77:1 at 25 °C. The highest 2-MAG of 98% was achieved after 4 h of incubation.



**Figure 2** Effect of organic solvent on acylglycerol production by immobilized lipase. The process was performed at a 5% water content, and glycerol was mixed with PO at a molar ratio of 6:1 in organic solvent (25 wt% of PO) at 40°C and 250 rpm for 24 h. Bars represent the standard deviation from triplicate determinations.

### 3.1.3. Effects of water content

Effects of water content on the enzymatic synthesis of glycerol and lipase varied 0-10%. The highest acylglycerol content (90.5%) was achieved included a water content of 8.0% (Fig. 3).
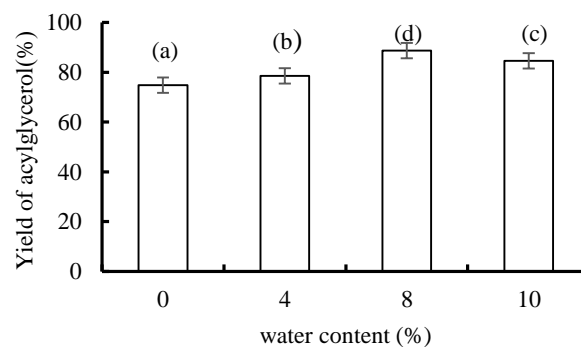


**Figure 3** Effect of water content on acylglycerol production by immobilized lipase. The process was performed at a water content of 0-10%, and glycerol was mixed with PO at a molar ratio of 6:1 in ethanol (25 wt % of PO) at 40 °C and 250 rpm for 24 h. Bars represent the standard deviation from triplicate determinations.

When increasing the water content (0–8%) of the enzyme, acylglycerol production increased. It has been reported that a hydrolysis reaction could not occur when a high-water content was used. A sufficient water content is essential to preserve enzyme stability and structure. Moreover, water is linked to the hydrolysis and esterification reaction as a reactant and a product, respectively. Therefore, the optimal

water content is essential to preserve the hydration layer around lipase and enzyme activity. In the current study, a high-water content resulted in a lower acylglycerol production. It may due to the high-water content catalyst a hydrolysis reaction rapidly than glycerolysis reaction. In previous studies [39-41] when water was added to the reaction in excess, the MAG yield did not increase; instead, it increased the formation of byproducts such as free fatty acids (FFAs). When the water content was higher than 10 wt%, low MAG was achieved because the high-water content caused hydrolysis reaction [42]. Moreover, the excess water surrounded the enzyme particle might prevent the substrate transport and substrate access, leading to particle aggregation [43,44].

### 3.1.4. Effects of the glycerol to oil ratio

Glycerol is generally used for the acylglycerol production because of its low cost. In the current study, molar ratios of glycerol to PO at 2:1 to 10:1 were assessed. Figure 4 showed that higher glycerol concentrations led to higher rates of acylglycerol production. The highest acylglycerol content was achieved when the molar ratio of glycerol to PO increased to 6:1.
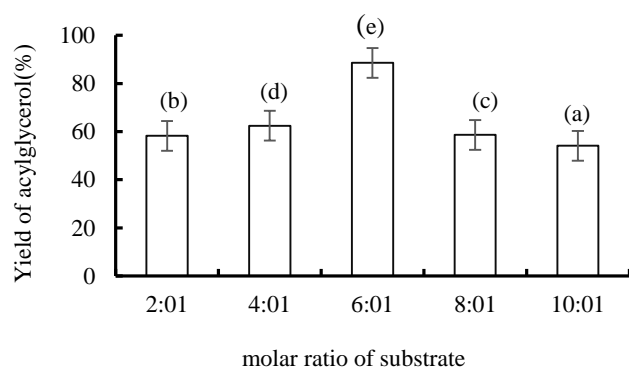


**Figure 4** Effect of glycerol to-oil (palm oil) ratio on acylglycerol production by immobilized lipase. The process was performed at a water content of 8%, and glycerol was mixed with PO at a molar ratio of 2:1 to 10:1 in ethanol (25 wt% of PO) at 40°C and 250 rpm for 24 h. Bars represent the standard deviation from triplicate determinations. Different letters indicate a statistically significant difference in the means ($p< 0.05$)

However, the acylglycerol yields decreased when the molar ratios of glycerol to PO further increased from 6:1 to 8:1 and 10:1. Although the initial rate of acylglycerol production decreased with increasing concentrations of glycerol at a molar ratio of glycerol to PO of 10:1. Decrease of acylglycerol production was due to the high concentration of glycerol, which resulted in a lower miscibility with PO. The molar ratio of glycerol to PO of 6:1 was the optimal value and the highest acylglycerol production (90.5%) was achieved. Therefore, the molar ratio of glycerol to PO of 6:1 was used for further experiments.

### 3.1.5. Effects of immobilized-enzyme loading

Effects of enzyme loading on acylglycerol production was assessed (Table 1).

**Table 1**. Effect of enzyme loading on acylglycerol production by immobilized lipase. The process was performed at a water content of 8%; glycerol was mixed with PO in a molar ratio of 6:1 in ethanol (25 wt % of PO), a 40 °C and 250 rpm for 24 h.

| Enzyme loading (%) | Acylglycerol yield (%) |
| --- | --- |
| 5 | $85.17 \pm 0.10$[a] |
| 10 | $86.94 \pm 0.05$[b] |
| 15 | $89.24 \pm 0.06$[c] |
| 20 | $91.17 \pm 0.06$[f] |
| 25 | $90.09 \pm 0.06$[e] |
| 30 | $89.49 \pm 0.04$[d] |

Values are mean Standard deviation (n=3) the different letters a,b,c,d,e,f in the same column denote significant differences ($p$ <0.05)

Results showed that acylglycerol increased when enzyme loading increased. Quantity of immobilized lipase varied 5-25% when PO and the molar ratio of glycerol to PO in ethanol were constant at 25% (w v$^{-1}$) and 6:1, respectively. Decrease of acylglycerol occurred when the quantity of immobilized lipase increased. It might be due to the high enzyme loading that created a faster hydrolysis than the glycerolysis. The optimal enzyme loading at 20% was the most sufficient quantity for acylglycerol and 91.17% of the product were achieved.

### 3.1.6. Effects of incubation temperature

Effects of incubation temperature varied 40–60 °C. The highest acylglycerol (91.2%) was achieved at 40 °C of incubation temperature. However, acylglycerol production did not significantly increase when temperature increased higher than 40 °C (Fig 5).
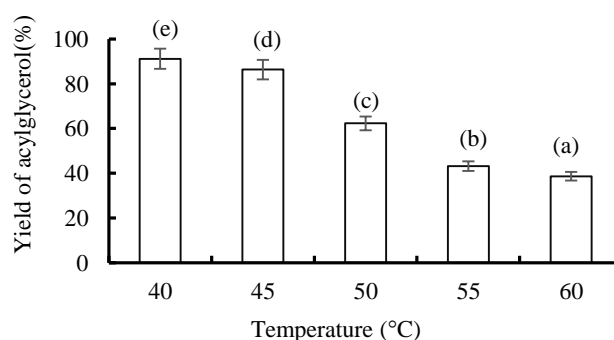


**Figure 5** Effect of incubation temperature on acylglycerol production by immobilized lipase. The process was performed at a water content of 8%, and glycerol was mixed with PO at a molar ratio of 6:1 in ethanol (25 wt % of PO) at 40 to 60°C and 250 rpm for 24 h. Bars represent the standard deviation from triplicate determinations. Different letters indicate a statistically significant difference in the means ($p< 0.05$)

This might be due to the increased reaction rate with increasing temperature. In contrast, when increasing the temperature from 40 ºC, acylglycerol yield decreased, most likely because the immobilized lipase was denatured by higher temperatures. The optimum conditions were as follows, 8% water content, glycerol mixed with 25 wt% PO in ethanol at a molar ratio of 6:1 at 40 °C for 24 h at 250 rpm. Then, acylglycerol was produced by CO and RBO under conditions similar to those used for PO acylglycerol. Acylglycerols from CO, RBO and PO were further characterized and used in oleogel production.

## 3.2. Characterization of acylglycerols produced with immobilized lipase

### 3.2.1. Analysis of acylglycerol production using gas chromatography-mass spectroscopy

Three acylglycerol samples from various oils (CO, RBO and PO) were analyzed using GC-MS. Results showed that acylglycerol from CO, RBO and PO contained MAG, DAG and TAG at 51, 30 and 8% of CO, 50, 25 and 12% of RBO and 85, 10 and 2% of PO, respectively. With PO as substrate, the maximum MAG yield of 85% was reached, followed by 51% with CO and 50% with RBO. Based on previous studies, oils most commonly used as raw materials for acylglycerol production included hydrogenated vegetable oils (soybean, rapeseed, cottonseed, coconut and rice bran oils [45]. Moreover, acylglycerol yield was highest (90%) from a glycerolysis reaction with non-hydrogenated oils, whereas the lowest yield 30% was achieved with hydrogenated oils (lard) [46-47]. A schematic diagram of enzymatic acylglycerol production using glycerolysis is shown in Table 2. Base on the studies on glycerolysis, the highest acylglycerol yield (87.4%) was achieved when using

camellia oil [48], followed by PO (85%, this study). Acylglycerol production with palm oil as substrate reached a maximum yield, resulting in production of acylglycerol with a high proportion of MAG (85%), this has not previously been reported for this acylglycerol production with immobilized lipases (Table 2).

Researchers have reported that plant, camellia, sunflower, echium, soybean and coconut oils can be used to produce high quantities of acylglycerol (53.7–87.4%) at 40–50°C (molar ratios of 4.5:1 to 6:1), respectively [28,34,35,48,49]. In 2020, Hobuss et al. [14] reported a high acylglycerol production (78%) using linseed oil as substrate at a molar ratio of 5:1 in NaOH at 130 °C for 12 h. This study successfully established production of acylglycerols from palm oil as a substrate with immobilized lipases. The economical production of acylglycerol is still a major challenge. Therefore, finding of potential raw materials and catalysts with high-efficiency with low-cost are still necessary.

### 3.2.2. Emulsion stability

Emulsion stability or a feature of the system to preserve its physiochemical characteristics. Emulsion stability is important in industrial uses of agriculture, food, medicine and energy [50].

In a study on emulsification in foods, food additives were used and various emulsifier types played important roles in creating a homogeneous appearance in products, resulting in improving quality of the products [51]. As various emulsifier types affect physical characteristics of the products in various ways, selecting the correct emulsifier type directly affect quality of the products [52]. In this study, acylglycerols from CO, PO and RBO were selected and effects of acylglycerol as an emulsifier was studied (Table 3).

Table 2. Comparison of acylglycerol production by various oils using different substrates.

| Substrate | Reaction condition | Product formed | characteristic | Reference |
|---|---|---|---|---|
| Camellia oil | mole ratio 6:1, at 50 °C | 87.4 | HPLC | [50] |
| Sunflower oil | 10-15 % (w w$^{-1}$) enzyme,mole ratio 4.5:1, at 40 °C and 700 rpm | 70 | HPTLC | [30] |
| Echium oil | mole ratio 9:1, at 40 °C and 200 rpm for 48 h | 80 | - | [43] |
| Soybean oil | mole ratio 10:1, water content of 5 wt%, at 45 °Cfor 12 h | 53.7 | - | [64] |
| Linseed oil | mole ratio5:1 in sodium hydroxide (NaOH), at 130 °C for 12 h | 76 | GC-IR | [14] |
| Palm oil | | 85 | GC-MS | This study |
| Coconut oil | mole ratio 6:1, water content of 8 wt%, at 40°C for 24 h | 51 | | |
| Rice bran oil | | 50 | | |

**Table 3.** The emulsion stability using different source of acylglycerol.

| Source of acylglycerol | Aqueous phase separation (ml) | | |
|---|---|---|---|
| | 5 minutes | 10 minutes | 15 minutes |
| No acylglycerol | $1.17^c \pm 0.04$ | $1.05^b \pm 0.02$ | $0.98^a \pm 0.01$ |
| CO | $0.33^c \pm 0.16$ | $0.36^b \pm 0.19$ | $0.59^a \pm 0.13$ |
| RBO | $0.26^a \pm 0.04$ | $0.38^b \pm 0.07$ | $0.56^c \pm 0.03$ |
| PO | $0.25^a \pm 0.18$ | $0.31^b \pm 0.20$ | $0.44^c \pm 0.13$ |

Values are mean Standard deviation (n=3) the different letters a, b, c, in the same row denote significant differences ($p$ <0.05), CO = Acylglycerol produced from coconut oil, RBO = Acylglycerol produced rice bran oil and PO = Acylglycerol produced palm oil

Emulsifier type and mixing time synergistically affect the emulsion stability. Use of acylglycerol from CO, PO and RBO as emulsifiers in oils resulted in decreased quantity of the separable liquids. This indicated that the emulsified oil included a higher emulsification stability, compared to that the non-emulsified oil with the same mixing time did. Increasing the mixing time resulted in improved stability of the oil-water emulsion added to acylglycerol, possibly due to the small quantity of liquid separating from the sample. This occurred because in the mixing process, emulsifier was added to the oil and water. This component included approximately 85% water before being added to the oil; thus, helping acylglycerol bind well with non-polar components. It did not fully functional as an emulsifier and when the mixing time increased, consistency of the emulsion was higher. However, increasing the mixing time with acylglycerol decreased emulsion stability. This might be because the acylglycerol added to the product acted well as an emulsifier. Within a short mixing time (5 min), acylglycerol could bind well with polar compounds. However, when the mixing time increased, water fragments might be dispersed in the emulsion, resulting in the oil losing its emulsion nature. Therefore, the addition of emulsifiers contributes to the stability of the emulsion of the oil and water. Mixing the samples with acylglycerol for 10 and 15 min improved stability of the emulsion, compared to the samples with no emulsifiers at a similar mixing time (Table 3). Regarding volume of the liquid separated from the three oil types, CO included the least emulsion stability, followed by RBO and PO.

### 3.2.3. Viscosity

Oil viscosity analysis revealed that emulsifier type and mixing time co-affected sample viscosity. Compared to the effects of the emulsifier at a similar mixing time, samples with the three emulsifiers included a higher viscosity than those with no emulsifiers. In addition to acting as a binder, emulsifiers facilitated dispersion of the water and oil parts. Emulsifiers could increase product viscosity [53]. Increasing the mixing time increased viscosity of the sample. In general, viscosity is important for emulsion systems. When a drop of fat slowly moves, aggregation of the fat droplets takes more time, resulting in a higher stability of the emulsion system

[54]. Table 4 shows results of the emulsion viscosity study. Increasing the mixing time resulted in increases in viscosity. In sample with emulsifiers of CO and RBO, viscosity increased slightly with increasing the mixing time. However, a longer mixing time resulted in significant increases in viscosity in samples with PO. At a mixing time of 5-10 min, emulsifier of PO created the best viscosity.

**Table 4.** The oil viscosity using different source of acylglycerol.

| Source of acylglycerol | Oil viscosity (Pa.s) | | |
|---|---|---|---|
| | 5 minutes | 10 minutes | 15 minutes |
| No acylglycerol | $0.42^b \pm 0.02$ | $0.35^a \pm 0.01$ | $0.53^c \pm 0.01$ |
| CO | $0.92^a \pm 0.02$ | $1.00^b \pm 0.15$ | $1.01^c \pm 0.02$ |
| RBO | $0.61^c \pm 0.01$ | $0.63^a \pm 0.01$ | $0.72^b \pm 0.20$ |
| PO | $0.66^a \pm 0.01$ | $0.67^b \pm 0.08$ | $0.93^c \pm 0.17$ |

Values are mean Standard deviation (n=3) the different letters a, b, c, in the same row denote significant differences ($p$ < 0.05), Oil viscosity was determined at 170 sec. CO = Acylglycerol produced from coconut oil, RBO = Acylglycerol produced rice bran oil and PO = Acylglycerol produced palm oil

### 3.3. Use of acylglycerol for oleogel production

Various oils (CO, RBO and PO) were used to prepare oleogel that formed coconut oil oleogel, rice bran oil oleogel and palm oil oleogel, respectively. Oleogel samples were prepared by dissolving acylglycerol mixture in CO, RBO and PO at concentration of 7 g oil and temperature of 90 °C with mild agitation (400 rpm) in a water bath under thermostat control. Samples were heated until acylglycerol was absolutely dissolved in the oil. Then, samples were cooled down to room temperature (25 °C) and stored at cold temperature (5 °C ±0.5) for 24 h. Physical characteristics of the acylglycerol oleogel were studied using SEM and FTIR.

### 3.3.1. Visual appearance of oleogels

Prepared oleogels were assessed for appearance and gel formation at room temperature (25 °C) within 1 h. Differe-nces in appearance and color of oleogels are shown in Fig 6. Yellow oleogels were seen from the productions of PO and RBO. However, whitish oleogels were achieved from CO. Different colors of the oleogels depended on the color and type of oil used in the reactions.
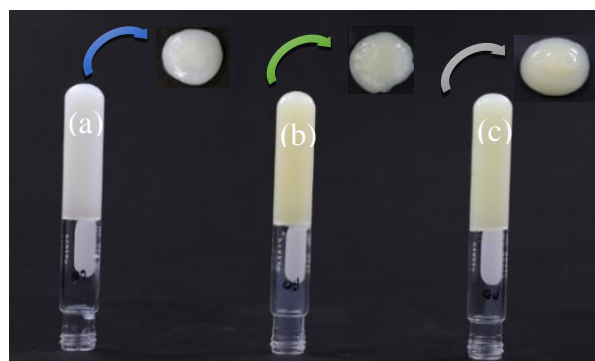


**Figure 6** Photograph of oleogels prepared with (a) coconut oil, (b) rice bran oil, and (c) palm oil.

Moreover, turbid oleogels revealed formation of large crystals of the gelation [55]. In addition, formation of a stable gel network occurred because no separation was investigated for the sample within 1 h at room temperature (25 °C). This result verifies the gel network formation and oil-binding capacity.

### 3.3.2. Physical characteristics of the acylglycerol oleogels using scanning electron microscopy

Internal network of the acylglycerol oleogels was assessed using SEM. Figure 7 shows 200 µm of small oil-filled pores of coconut oil oleogel, rice bran oil oleogel and palm oil oleogel. Moreover, SEM micrographs showed that the molecules were able to form self-assemblies into inverse bilayers when acylglycerol was added into the oils. However, the optical microscopy showed that coconut oil oleogel

included a large cavity, compared to that the other oils did (Fig. 7), indicating that a continuous network was not seen in coconut oil oleogel [56-59].

### 3.3.3. Characterization of the acylglycerol oleogels using Fourier transform infrared spectroscopy

Figure 8 shows ART-FTIR spectra of the oleogel samples, demonstrating a band in the acylglycerol. According to den Adel et al. [60], medium-intensity bands at 3570-3450 cm$^{-1}$ revealed intermolecular and intramolecular hydrogen bonds, respectively. Figure 8 shows intermolecular hydrogen bonds in the oleogels. This might be due to the availability of -OH groups in the acylglycerol molecules, which could form hydrogen bonds within the molecules [61]. Relatively, presence of the hydrogen bonds was shown in oleogel samples.
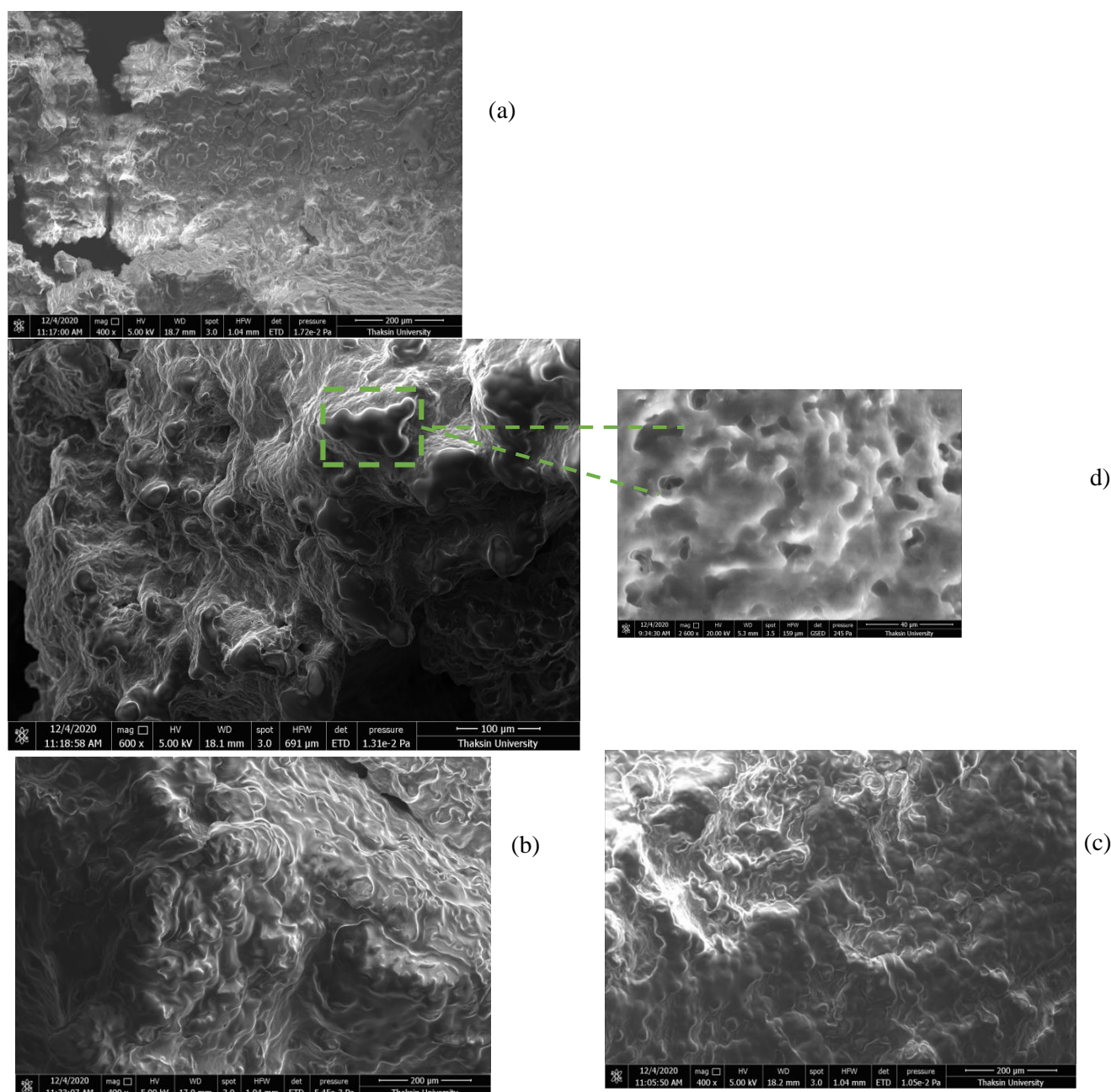


**Figure 7** SEM images of coconut oil oleogel (a, d), rice bran oil oleogel (b), and palm oil oleogel (c).
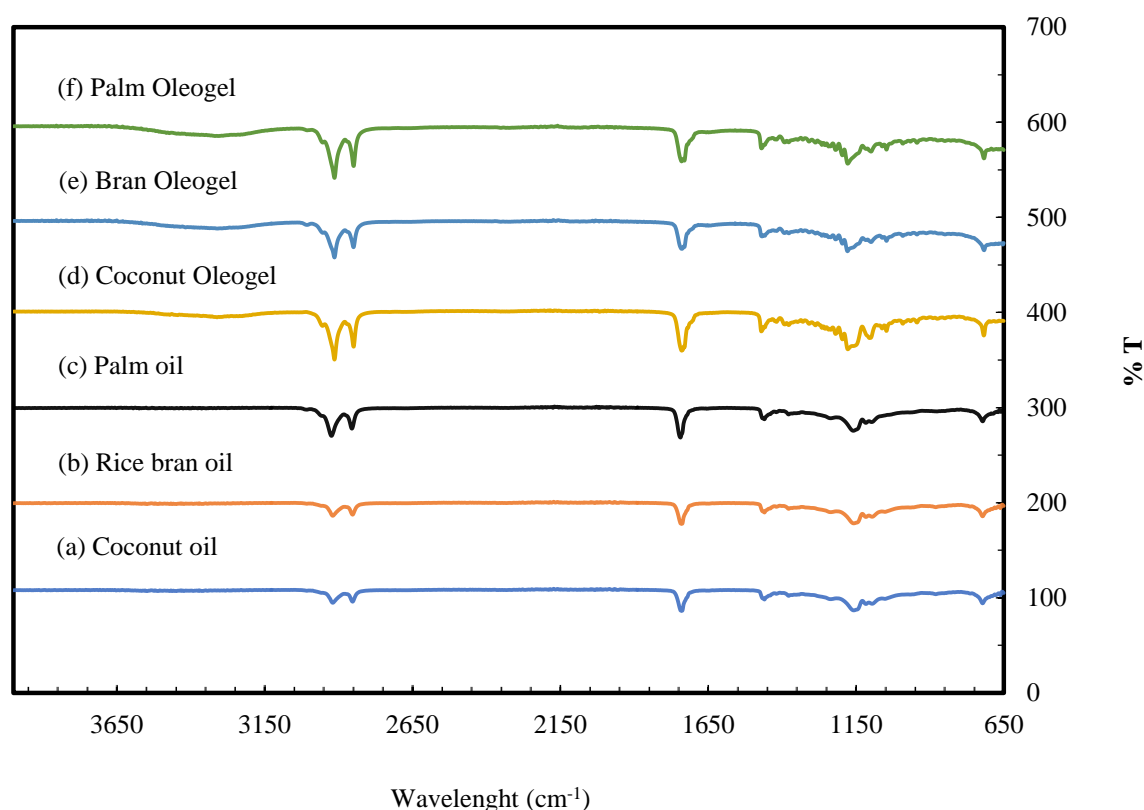
**Figure 8** FTIR spectrum of the acylglycerol sample of coconut oil (a), rice bran oil (b), and palm oil (c) and oleogel samples of coconut oil oleogel (d), rice bran oil oleogel (e), and palm oil oleogel (f).

## 4. Conclusion

In the present study, optimized conditions to achieve maximum acylglycerol yields were assessed using CO, RBO and PO with glycerol as substrate and immobilized lipase as catalyst. Effects of various parameters on the glycerolysis process were assessed as well. The highest acylglycerol yield (91.15%) was achieved through the following optimal conditions: glycerol-to-palm oil ratio of 6:1, enzyme loading of 20%, water content of 8%, reaction temperature of 40 °C and time of 24 h. Addition of acylglycerol could help improve stability as well as decreasing the viscosity limitation. Based on the results from other studies, acylglycerol can be produced from biodiesel glycerol and used as substrate for oleogels. Moreover, the oleogels showed possibility of using in foods. In this study, use of biodiesel glycerol as a substrate of acylglycerol using immobilized lipases seemed promising since high acylglycerol quantities were produced. Immobilization created best performance of the lipase use. However, the cost of lipase serves as a major limitation for its use in industries. Therefore, the enzyme cost should decrease using recovered and reused enzymes. Investigation of novel lipases from low-cost sources such as plants or microorganisms is recommended. Moreover, increases in quality of oleogels to food-grade oleogels and studies on food-grade oleogels should be facilitated with further details

## 5. Acknowledgements

## 6. Conflict of Interest

The authors report no conflicts of interest.

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# استفاده از گلیسرول خالص شده از کارخانجات بیودیزل به‌منظور تولید آسیل گلیسرول و کاربرد آن در مواد غذایی

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# چکیده

**سابقه و هدف:** بیودیزل سوخت مایع است که بخوبی شناخته شده می‌باشد. با این‌حال، هنگام تولید بیودیزل مقادیر زیادی گلیسرول به‌عنوان فرآورده جانبی تولید می‌شود. اگر این ماده به‌عنوان رشدمایه[1] برای فرآورده‌های با ارزش افزوده مانند مونوآسیل گلیسرول مورد استفاده قرار گیرد، تداوم اقتصادی فرایند بیودیزل می‌تواند بهبود یابد. مونوآسیل گلیسرول کاربردهای گوناگونی دارد. با این‌حال، کاربرد این ماده می‌تواند به‌عنوان رشدمایه برای اولئوژل هنوز با چالش روبروست. از این‌رو، هدف این مطالعه بررسی تولید بهینه آسیل گلیسرول توسط لیپاز تثبیت شده با استفاده از گلیسرول حاصل از فرایند بیودیزل و روغن‌های گوناگون گیاهی به عنوان رشدمایه بود. علاوه بر این، کاربرد آسیل گلیسرول برای تولید اولئوژل مورد مطالعه قرار گرفت.

**مواد و روش‌ها:** در ابتدا، گلیسرول از کارخانجان بیودیزل جمع‌آوری و با استفاده از چرخه‌های مکرر اسیدی کردن خالص شدند. گلیسرول خالص شده و انواع گوناگون روغن‌های گیاهی، شامل روغن‌های نارگیل، سبوس برنج و پالم، برای تولید آسیل گلیسرول با استفاده از کاتالیست لیپاز تثبیت شده مورد استفاده قرار گرفتند. سپس آسیل گلیسرول‌های به‌دست آمده از هر روغن گیاهی جمع‌آوری و به‌عنوان رشدمایه برای اولئوژل استفاده شد. ویژگی‌های آسیل گلیسرول با استفاده از روش‌های استاندارد کروماتوگرافی گازی و طیف‌بینی مادون‌قرمز تبدیل فوریه[2] تعیین شد. علاوه بر این، ساختار و توسعه اولئوژل با استفاده از طیف‌بینی مادون‌قرمز تبدیل فوریه و میکروسکوپ الکترونی روبشی[3] مورد بررسی قرار گرفت.

**یافته‌ها و نتیجه‌گیری:** بیشترین میزان بازده (۹۱/۱۵٪) در شرایط ۲۰٪ بارگذاری آنزیم، نسبت ۶ به ۱ گلیسرول به روغن پالم، ۸٪ آب، ۴۰°C درجه واکنش و ۴ ساعت زمان واکنش به‌دست آمد. آسیل گلیسرول به‌دست آمده از روغن پالم شامل مونو، دی و تری آسیل گلیسرول‌ها در مقادیر به‌ترتیب ۸۵/۰، ۱۰/۰ و ۲/۰ درصد بود. نتایج این مطالعه نشان داد که نوع روغن بر کیفیت اولئوژل تاثیری ندارد و تمام اولئوژل‌ها ویژگی مناسب برای استفاده در مواد غذایی را دارند.

**تعارض منافع:** نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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1 substrate

2 Fourier transform infrared spectroscopy or FTIR

3 scanning electron microscope or SEM