Determination of optimum conditions to produce stearoyl glycerol enzymatically

Anna Roosdiana,1* Chanif Mahdi,1 Sutrisno,1 Zahza Fatika Rahma1
1Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

*Corresponding author: aroos@ub.ac.id

Abstract. Stearoyl glycerol is the result of the esterification of glycerol with stearic acid enzymatically using immobilized lipase as catalyst. The lipase is immobilized with chitosan matrix with entrapment method. This study aims were to determine the optimum condition of esterification of glycerol with stearic acid based on reaction time and temperature, and reactant mole ratio. The esterification reaction was carried out by varying reaction time (6, 12, 18, 24, 30) hours, reaction temperature (30, 35, 45, 50, 55) °C, and mole ratio of stearic acid : glycerol (1: 1, 1: 2, 1: 4, 1: 6, 1: 8), the optimum conditions were achieved through conversion percentage. The produced ester was identified by FTIR spectrophotometer and characterized by the value of Hydrophilic-Lipophilic Balance (HLB). The results showed that optimum conditions on reaction time 24 hours, reaction temperature 45°C and mole ratio of stearic acid:glycerol (1:2) produced stearoyl glycerol with conversion percentage of 41.04% and HLB values of 5.01 that was included in the w/o emulsifier type. In addition, the FTIR spectra showed a strong absorption at wave numbers 1703.03 cm\(^{-1}\) (C=O), 1043.02 cm\(^{-1}\) (C-O), and 3402.20 cm\(^{-1}\) (O-H) which indicated the characteristic absorption of stearoyl glycerol.

1. Introduction
Stearoyl glycerol is emulsifier which included in a non-ionic surfactant and has low hydrophilic-lipophilic balance (HLB. The stearoyl glycerol is widely used in food and feedstuff, pharmaceutical formulations, cosmetics, plasticizers and drug delivery systems [1]. Indonesia requires 95000 tons emulsifier annually and 45000 tons emulsifier is still imported. 75% of total emulsifier need derived from mono and diglyceride [2]. The stearoyl glycerol can be produced from esterification of glycerol with stearic acid. The glycerol is a by-product on biodiesel production which is obtained at high concentration in a weight ratio of 1/10 (glycerol/biodiesel) [3], whereas stearic acid can be achieved from natural oil.

Stearoyl glycerol is conventionally produced by esterification of glycerol with stearic acid and transesterification of glycerol with natural fats and oils or stearic acid methyl ester. These reactions need homogeneous catalysts which are either basic (such as metal hydroxides, methyl oxides and inorganic carbonates) or acidic (such as phosphoric acid, sulfuric acid and organic sulfonic acid). However, this conventional method has a drawback such as low selective resulting in the mixture of mono, di and triglyceride. Other synthetic methods for producing stearoyl glycerol have utilized lipase- catalysed reactions [1].
Lipase has function as biocatalyst to hydrolyze triglyceride become free fatty acid and glycerol, but in the free water condition, the lipase function as biocatalyst in esterification reaction. In order to enhance the lipase stability, being reused and controllable reactions, lipase is immobilized in certain matrices [4]. Immobilization of lipase can use chitosan as matrix, since chitosan is biodegradable, low cost, easy to handle, high affinity to protein, hard structure, inert, low density lead to chitosan can bind free enzyme and enable to stabilize the high catalytic activity.

There are some factors which can affect the activity of immobilized lipase, such as mole ratio of reactant, temperature, reaction times. The lipase is currently used in transesterification reaction, for example transesterification of olive oil and polyethylene glycol to produce polyethylene glycol di oleate. The ester is maximally produced at the mole ratio of olive oil and polyethylene glycol 1:9 within 4 hours of incubation at 40 °C resulting in 97.62% of ester conversion [5]. The lipase is also used as biocatalyst in esterification reaction. Candida antartica lipase has been used in the esterification of sorbitol with some fatty acids such as oleic acid, palmitic acid and lauric acid and the optimum condition of esterification occurred at 40-50°C in 48 hours [6].

In the present study, esterification of glycerol with stearic acid used immobilized lipase in chitosan as a catalyst. Determination of optimum condition of esterification reaction based on reaction times, reaction temperature and the mole ratio of glycerol and stearic acid which showing the highest conversion percentage of glycerol to stearoyl glycerol. The resulted stearoyl glycerol was identified through FTIR spectrum and determined its emulsifier type based HLB value.

2. Materials and Methods

2.1. Chemicals and Instrumentations.
Analytical grade stearic acid (C_{18}H_{36}O_2), sodium hydroxide (NaOH), glycerol (C_{3}H_{8}O_3), glacial acetic acid (CH_3COOH), pyridine (C_5H_5N), benzene (C_6H_6), t-butanol (C_4H_10O), Sodium tripolyphosphate (Na_5P_3O_10, 367.86g/mol) were products of Merck Germany. Lipase from Candida rugose, lyophilized powder was purchased from Sigma Aldrich and used as received. The instruments used in this research were spectrophotometer FTIR (8400 S Shimadzu), Analytical balance (Mettler Toledo AL 204), oven (Memmert).

2.1.1. Preparation of immobilized lipase in chitosan
Chitosan (0.625g) was dissolved in 25 mL of 3% glacial acetic acid solution, the mixture was homogenized with magnetic stirrer. Lipase (0.5g) was mixed with 12mL of 2.5% chitosan solution, then the lipase mixture was added drop-wise by using syringe to 10mL of 3% sodium tripolyphosphate solution and allowed to stand for 120 minutes. The droplets (immobilized lipase) were separated by filtration, dipped in phosphate buffer pH 8 and stored in refrigerator.

2.1.2. Esterification of glycerol with stearic acid enzymatically.
Determination of optimum reaction time: Glycerol (0.08g) and stearic acid (0.28g), buthanol (10mL) and immobilized lipase (0.1 g) were put in Erlenmeyer flask 100 mL. The mixture was incubated in various reaction time of (6, 12, 18, 24, 30) hours at temperature 50°C. At the end of the reaction, 10mL ethanol, 3 drops of phenolphthalein indicator were added to the mixture and titrated with 0.1N NaOH alcoholic solution until colour change from colourless to pink. Each variation of reaction time in this procedure was carried out in triplicates. The conversion percentage of glycerol to stearoyl glycerol ester was calculated based on the mole number of reacted stearic acid divided by the mole number of glycerol and multiplied by 100%.

Determination of optimum reaction temperature: Esterification was carried out at optimum reaction time and various reaction temperature of ((30, 35, 45, 50, 55)°C. The procedures were similar with the procedures of optimum reaction time determination.
Determination of mole ratio of reactants: Esterification was carried out at optimum reaction time and temperature. The mole ratio of glycerol and stearic acid were 1:1, 1:2, 1:4, 1:6, 1:8 (mass of glycerol was 0.08g and the mass of stearic acid was 0.28, 0.56, 1.12, 1.68 and 2.24 g). The procedures were similar with the procedures of optimum reaction time determination.

2.1.3 Identification of resulted stearoyl glycerol by FT-IR spectrophotometer.
Glycerol, stearic acid and stearoyl glycerol ester were analyzed using FTIR to determine its functional groups, glycerol was dropped to NaCl plate, while stearic acid or stearoyl glycerol each 0.05g was ground with KBr (0.1g) and molded as KBr pellets. In order to obtain spectra, all samples were measured their transmission at wave numbers 4000-400 cm⁻¹.

2.1.4 Analysis of Hydrophilic-lipophilic Balance (HLB)
Determination of HLB value was conducted through water number method. The resulted stearoyl glycerol was weighed 0.1g, then dissolved in 2.5mL of pyridine-benzene mixture (95:5). The mixture was titrated by distilled water until appearing of permanent turbidity. This procedure was also applied for various emulsifier which known its HLB value, then standard curve was made from HLB value vs used water volume of titrant in order to obtain linear regression equation. The HLB of resulted stearoyl glycerol was achieved by inserting volume of the water used into linear regression equation [7].

2.2 Analysis of data
The data of conversion percentage at different reaction time, reaction temperature and mole ratio of reactants were analyzed statistically using one-way Analysis of Variance (ANOVA), followed by Tukey test multiple comparison tests. The level for statistically significant was set at a P value <0.05. The analysis used SPSS 23.0 for windows, while the data of FTIR spectra and HLB value were analyzed descriptively.

3. Results and Discussion
Enzymatic esterification reaction is affected by many factors which includes substrates and enzyme concentration, the molar ratio, the reaction pH –value and temperature, mixing rates and the water level [8]. Esterification reaction in this work was carried out using butanol as solvent. The optimum reaction time for esterification of glycerol with stearic acid took place with in 24 hours resulting in 2.47% of conversion percentage. As can be seen from Figure 1 that conversion percentage of glycerol to stearoyl glycerol increased at 6-24 hours and it decreased at 30 hours due to the esterification reaction produced water as a byproduct. Increasing the amount of water can affect the lipase activity in organic solvent and shift from esterification reaction to hydrolysis reaction. The increase of water activity declines the rate reaction and the yield. Consequently, controlling the amount of water is required to increase the yield of esterification reaction.

Besides the reaction time, temperature also affects the esterification reaction. As shown in Figure 2. At reaction temperature 30 to 45°C, the conversion percentage of glycerol to ester increased with increasing reaction temperature due to the increase of lipase activity. Optimum reaction temperature occurred at 45°C resulting in 5.33% of conversion percentage. It decreased at 50 and 55°C because the high temperature will inactivate lipase through conformation change of enzyme.

The esterification reaction is also influenced by the mole ratio of reactants. It can be seen from Figure 3. The conversion percentage of glycerol to ester occurred at mole ratio of 1:6. However, at mole ratio of 1:8 that showed sharply decrease. The enzymatic esterification reaction follows the Ping pong Bi-Bi mechanism [9]. The esterification reaction undergoes two mechanisms, namely acylation and deacylation. In the acylation stage, O atom of catalytic serine residue will bind carbonyl group of stearic acid to form first intermediate. Then, the intermediate undergoes dehydration with releasing H₂O to form acyl-enzyme complex. At the de-acylation stage, the acyl-enzyme complex react with OH- primary group of glycerol to form second intermediate which then release a steroyl glycerol as final product and serine residue returns to its original lipase. At mole ratio of 1:8, the conversion percentage decreased
that might be caused by the ability of lipase to react with stearic acid has been saturated and produced maximum number of acyl enzyme, however the acyl–enzyme is inhibited to react with glycerol molecule because of too much stearic acid in surrounding leading to less ester produced.

Figure 1. The effect of reaction times to conversion percentage of glycerol to stearoyl glycerol at 50 °C and the mole ratio of glycerol and stearic acid (1:1)

Figure 2. The effect of reaction temperature to conversion percentage of glycerol to stearoyl glycerol within 24 hours and the mole ratio of glycerol and stearic acid (1:1)

Figure 3. The effect of mole ratio of glycerol and stearic acid to conversion percentage within 24 hours at 45°C

Identification of functional groups of glycerol, stearic acid and stearoyl glycerol through FTIR spectra was conducted to approve the resulted ester. As shown in Figure 4. FTIR spectra of glycerol, stearic acid and stearoyl glycerol showed difference in absorption of several functional groups, such as C=O, C-O, O-H, aliphatic CH and others. The absorption of O-H group of stearoyl glycerol is in the wave number 3402.20 cm⁻¹, while the glycerol spectra is in wave number 3382.91 cm⁻¹. The absorption intensity of O-H functional groups of glycerol is higher than stearoyl glycerol due to glycerol has three OH functional groups and stearoyl glycerol has two OH functional groups. Moreover, the absorption intensity of CH of stearoyl glycerol is slightly sharper at wave numbers 2920.03 cm⁻¹ and 2850.59 cm⁻¹ compared to absorption intensity of CH of stearic acid in wave numbers 2818.10 and 2848.67 cm⁻¹. In addition, the formation of stearoyl glycerol can be approved from the appearance of absorption band of C=O functional group at wave number 1703.03 cm⁻¹ and absorption band of C-O functional groups at wave numbers 1042.42 cm⁻¹.

The HLB value is a ratio value of hydrophilic lipophilic groups in a surfactant. The longer the lipophilic chain the higher HLB value. The HLB value below 6 is used as emulsifier in W/O system, while the HLB value is greater than 9 can be used as emulsifier in O/W system [10]. Stearoyl glycerol is measured based on water titration method which need various surfactants with different HLB values as standards. Glycerol is hydrophilic compound that will attract water molecule which is polar and
nitrogen ions of pyridine which is non-polar, whereas stearic acid in stearoyl glycerol will attract nonpolar benzene molecule and aromatic heterocyclic rings of pyridine molecule. The end point titration is indicated by appearing the permanent turbidity. In this situation, the solution is saturated and the stearoyl glycerol cannot longer bind to water molecule or solution of pyridine and benzene. The standard curve was made from HLB value number of water used resulting in linear regression equation of Y = 0.2785 - 2.2815. Titration of stearoyl glycerol need 2.9 mL water, then the water number is inserted to that linear regression equation and obtained the HLB value of stearoyl glycerol 5.01. Thus, stearoyl glycerol is included in the water in oil (W/O) emulsifier type.

Figure 4. FTIR Spectra of Glycerol (---), stearic acid (----), and stearol glycerol (-----)

4. Conclusion
In this work, the synthesis of stearoyl glycerol from glycerol and stearic acid was studied. In 24 hours of reaction time, 45°C, mole ratio of glycerol and stearic acid (1:6) and immobilized lipase resulted maximum conversion percentage of glycerol to stearoyl glycerol. The resulted stearoyl glycerol was identified through FTIR spectra which showed peaks at waves number 3402.20 cm⁻¹ as functional group of (O-H), 2929.03 and 2850.59 cm⁻¹(C-H), 1703.03 cm⁻¹ (C=O), 1043.02 cm⁻¹ (C-O). In addition, The HLB value of stearoyl glycerol is 5.01 that is included in w/o emulsifier type. Further study should be made in order to produce high conversion percentage by limiting the water level in the reaction.

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