Enzymatic Synthesis of Glycerol-Coconut Oil Fatty Acid and Glycerol-Decanoic Acis Ester as Emulsifier and Antimicrobial Agents Using Candida rugosa Lipase EC 3.1.1.3

Sri Handayani, Ayu Tanissa Tamara Putri, Siswati Setiasih, Sumi Hudiyono*

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Indonesia

* sumi.hudiyono@sci.ui.ac.id

Abstract. In this research, enzymatic esterification was carried out between glycerol and fatty acid from coconut oil and decanoic acid using n-hexane as solvent. In this reaction Candida rugosa lipase was used as biocatalyst. Optimization esterification reaction was carried out for parameter of the substrate ratio. The mmol ratio between fatty acid and glycerol were used are 1:1, 1:2, 1:3, and 1:4. The highest conversion percentage obtained at the mole ratio of 1:4 with the value of 78.5% for the glycerol-decanoic acid ester and 55.4% for the glycerol coconut oil fatty acid ester. Esterification products were characterized by FT-IR. The FT-IR spectrum showed that the ester bond was formed as indicated by the wave number 1750-1739 cm⁻¹. The esterification products were then examined by simple emulsion test and was proved to be an emulsifier. The glycerol-coconut oil fatty acid ester produced higher stability emulsion compared with glycerol decanoic ester. The antimicrobial activity assay using disc diffusion method showed that both glycerol-coconut oil fatty acid ester and glycerol-decanoic ester had the ability inhibiting the growth of Propionibacterium acnes and Staphylococcus epidermidis. Glycerol-decanoic ester shows higher antimicrobial activity than glycerol-coconut oil fatty acid ester.

1. INTRODUCTION

Coconut oil is widely used for food, industrial applications, health, and disease prevention [1]. This oil contains various fatty acids such as lauric acid, palmitic acid, decanoic acid (capric acid), myristic acid, etc [2]. Production of derivative products (oleochemicals) from coconut oil can provide added value and higher profits than just exporting crude coconut oil. In oleochemistry coconut oil can be converted into various products to get higher economic value, such as mono- and diglyceride. Mono- and diglycerides are highly valued oil diversification products with high market prospects. Those glycerides are needed in industry such as food and pharmaceutical, cosmetics, and cleaning products as surfactants or emulsifiers. Fatty acid monoesters are also known to have antibacterial activity against both gram-positive and gram-negative bacteria [3].

Monoglyceride (MAG) is an ester compound consisting of one glycerol molecule binding to one fatty acid molecule [4]. Fatty acid monoesters are also known to have the highest antibacterial activity against both gram-positive and gram-negative bacteria [3]. In addition, glycerol esters with short or medium chain fatty acids tend to have greater antimicrobial activity compared to glycerol esters with long-chain fatty acids [5].

The study of esterification using Candida rugosa lipase EC 3.1.1.3. between simple saccharides and fatty acids [2, 6] and trans and interesterification reactions of palm oil has been conducted [7, 8]. In this study, glycerol esters were synthesized by esterification reaction between glycerol and coconut oil fatty acid and oleic acid using Candida rugosa lipase as biocatalyst. In the presence of organic solvent and small amount of water, the lipase may act as biocatalyst in an esterification reaction [9]. The glycerol-fatty acid ester produced was then examined its emulsifier and antimicrobial activity.
Lipase is a hydrolase class of enzyme that catalyzes the triglyceride hydrolysis produces fatty acids and glycerol. On certain conditions, lipases can also catalyze the reverse reaction of hydrolysis, namely the esterification reaction. Esterification reactions catalyzed by lipase occur in the system with small amount of water (essential water) [10]. Moreover solvent hydrophobicity affects the amount of essential water needed by an enzyme for its catalytic activity. The use of non-polar solvent was chosen so that the lipase tended to catalyze the esterification reaction [11, 12].

In performing its function as a catalyst, an enzyme must be in a good condition to support its catalytic activity. A study on catalytic activity of enzymes in organic solvents showed that solvents with log P values between 2 and 4 can maintain enzyme activity and stability [13]. Based on earlier research, esterification was performed using n-hexane as solvent which has a log p value of 3.5 [2].

2. MATERIALS AND METHODS

2.1 Materials

Materials used in this study were Candida rugosa Lipase (2.45 U/mg) obtained from Sigma-Aldrich, coconut oil, commercial glycerol, decanoic acid, potassium hydroxide, 96% ethanol, hydrogen chloride, sodium hydroxide, aquades, Na₂SO₄ anhydrate, n-hexane, phosphate buffer pH 8, phenolphthalein, eosin, clindamisin, DMSO 10%, nutrient broth and nutrient agar, Propionibacterium acnes and Staphylococcus epidermidis.

2.2 Methods

2.2.1 Hydrolysis of Coconut Oil

Hydrolysis of coconut oil was conducted by mixing 20 g of coconut oil and 100 mL KOH 1 M in ethanol and heated by reflux system for 1 hour at 62±2 °C. Coconut oil fatty acid was then extracted using n-hexane and continued by evaporation to remove the n-hexane [6].

2.2.2 Esterification

Esterification was carried out by mixing glycerol, coconut oil fatty acid, n-hexane as solvent, and Candida rugosa Lipase as a catalyst. The ratio of glycerol to fatty acid used were varied, ie 1:1, 2:1, 3:1, and 4:1 (mol / mol). The ratio solvent to substrate used was of 1: 1 (v / v substrates), while the enzyme used was 5% of the total mass of the substrate (w/w substrates). The mixture was then incubated in a horizontal incubator shaker at 200 rpm, 37 °C for 18 hours. The reaction was stopped terminated by heating at ± 80 °C. The mixture was then centrifugated at 3400 rpm for 15 minutes. Three layers were formed and then separated [2]. The same process were repeated performed using decanoic acid.

2.2.3 Determination of Conversion Percentage

The conversion percentage was determined by titrating the residual fatty acid in n-hexane using 0.1 N NaOH and phenolphthalein as indicator [2].

2.2.4 Characterization of Esterification Product using FT-IR

The esterification product, glycerol, fatty acid obtained from coconut oil hydrolysis, and decanoic acid were characterized using FT-IR.

2.2.5 Simple Emulsifier Test and Determination of Emulsion Type

Simple emulsifier test was performed by mixing 0.1 g esterification product, oil, and water according to Table 1. The mixtures were shaken and the emulsion stability was observed.
**Table 1 Variations of Oil and Water in the Making of Emulsions**

| Type of emulsion | Water (mL) | Oil (drops) |
|------------------|------------|-------------|
| Oil in Water     | 3          | 5           |
| Water in Oil     | 5          | 3           |

The emulsion type determination was performed by mixing a drop of emulsion and eosin on an object glass. The mixture was then observed under microscope to determine whether the emulsion formed is oil in water (o/w) or water in oil (w/o) emulsion.

2.2.6 *Antimicrobial Activity Assay*

Antimicrobial activity assay was performed using disc diffusion method. An aliquot of 200 μL of *Propionibacterium acnes* suspension with cell density of 1 x 10⁸ cells/mL was aseptically mixed by 20 mL of nutrient agar in a sterile petri dish. The medium was then allowed to harden. The sterile paper disc (6 mm in diameter) was placed on top of the medium and 4 μL sample was dropped onto this. The medium was then incubated at 37 °C for 24 hours. The positive control used was Clindamycin 0.5% and the negative control was DMSO. The clear areas around the paper discs were measured. The same assay was performed using *Staphylococcus epidermidis*.

3. RESULT AND DISCUSSION

3.1. Hydrolysis of Coconut Oil

The hydrolysis reaction was carried out using sodium hydroxide in ethanol as catalyst. The function of ethanol is to lower the polarity difference between KOH and oil which in the end permits the reaction. These fatty acids obtained from this process were used for esterification. Yield percentage of coconut oil hydrolysis was 94.9%.

3.2. Determination of Conversion Percentage

The product obtained from esterification reaction formed an emulsion system. To break the emulsion system, the mixture was centrifuged. The three layers formed were separated and the top layer was then used for conversion percentage determination.

The conversion percentage of esterification using various reactant mole ratios is shown in Figure 1. The conversion percentage increases with the increasing mole of glycerol. The amount of glycerol used was higher than fatty acids, thus the ester formed tends to be mono- or diglycerides.

The esterification reaction is an equilibrium reaction following the Le Chatelier principle. In order to improve the product of the reaction, excess reactants were used therefore the equilibrium will lead to the formation of product. The highest conversion percentage was achieved at mole ratio fatty acid to glycerol 1: 4 with the value of 55.4% using the coconut oil fatty acid and 78.5% using oleic acid as reactant.
Figure 1. The Curve of Mole Variation vs Conversion Percentage

3.3 Characterization of Esterification Product using FT-IR

IR spectrum of Glycerol, fatty acids from coconut and palm oil, coconut oil fatty acid ester, Decanoic acid, and Decanoic ester are shown in Figure 2.

FT-IR spectrum of glycerol showed an absorption peak at wave number 3650-3200 cm\(^{-1}\) for -OH functional group, 2931 cm\(^{-1}\) for C-H group, and 2877 cm\(^{-1}\) for -CH\(_2\) functional group (Figure 2.a). While FT-IR spectrum of coconut oil fatty acid showed there were absorption peaks at wave number 3000-2850 cm\(^{-1}\) indicating the presence of C-H functional group, 3000-2700 cm\(^{-1}\) which is a characteristic for CH\(_2\) and CH\(_3\) functional group, 1714 cm\(^{-1}\) for the C = O group (Figure 2.b and 2.d). Figure 2.c and 2.e showed that there are absorption peaks at wave number 3650-3200 cm\(^{-1}\) that indicate not all the O-H groups of glycerol were esterified. In addition there was an absorption peak at wave number 1748 cm\(^{-1}\) which indicated the
presence of –C=O ester functional group. Therefore, it can be concluded that the ester product was successfully produced.

3.4 Simple Emulsifier Test

The simple emulsifier test showed that emulsion formed using glycerol-coconut oil fatty acid ester as emulsifier more stable than using decanoic ester. The emulsion system formed stable up to 24 hours.

The simple emulsion type test showed that glycerol-decanoic ester has the ability to form water in oil (w/o) and oil in water (o/w) emulsion. But the w/o type formed was more stable than o/w type. For glycerol-coconut oil fatty acid ester tends to show the ability to form w/o emulsion. Figure 4 shows the microscopic photograph of the emulsion. The emulsion formed was added with eosin, a red dye which is soluble in water, so the water phase will be red in color and easy to be observed and distinguished from the oil phase [2]. Both glycerol-decanoic and glycerol-coconut oil fatty acid ester have better ability to form a water in oil (w/o) emulsion type. This can be seen from the formation of pink droplets while the surrounding was yellow. The droplet formed was the water phase and the light yellow environment around pink droplets was oil.

![Figure 3](image1.png)

**Figure 3** Simple emulsion test results

a) The oil in water (o/w) mixture + decanoic ester (left) and oil in water (o/w) mixture (right),

b) water mixture in oil (w/o) mixture + decanoic ester (left) and water in oil (w/o) mixture (right),

c) water in oil (o/w) mixture + coconut oil fatty acid ester, d) water in oil mixture

![Figure 4](image2.png)

**Figure 4** The Observed Emulsion Types with Microscopes

a. Water emulsion (w/o) using Glycerol-Decanoic ester

b. Water in oil emulsion (w/o) using Glycerol-Coconut Oil Fatty Acid ester

The type of emulsifier is influenced by the type and size of molecules of hydrophobic and hydrophilic part and described as HLB (Hydrophilic to Liophilic Balance) value. Emulsifiers with low HLB values (3-6) produce water-in-oil emulsions, whereas high HLB (8-18) produces an oil-in-water emulsion [14]. However, the glycerol-decanoic and glycerol-coconut oil fatty acid esters which tend to form monoester had a low HLB [15].
3.5 Antimicrobial Assay

Bacteria used in this study were *Propionibacterium acnes* and *Staphylococcus epidermidis*. *Staphylococcus epidermidis* is normal flora found in humans, while *Propionibacterium acnes* is acne-causing bacteria. Table 2 shows the antimicrobial activity of the ester products. Glycerol, n-hexane, and DMSO were also tested as control. Table 3 shows the effectiveness classification of antimicrobial substances [16].

The antimicrobial assay showed that glycerol-decanoic and glycerol-coconut oil fatty acid esters had middle category against *P. acnes* at concentrations of 75%. At concentrations 50% Glycerol-decanoic ester showed showed middle antimicrobial activity against *S. epidermidis*, while glycerol-coconut oil fatty acid ester up to concentration 75% showed weak activity against *S. epidermidis*.

| Sample                      | Concentration (%) | Inhibit zone diameter (mm) |
|-----------------------------|-------------------|---------------------------|
|                             | P. acnes          | S. epidermidis            |
| Glycerol-Decanoic Ester     |                   |                           |
| 25                          | 14 weak           | 14 weak                   |
| 50                          | 15 weak           | 16 medium                 |
| 75                          | 16 medium         | 16 medium                 |
| 100                         | 16 medium         | 17 medium                 |
| Glycerol-Coconut Oil Fatty Acid Ester |               |                           |
| 30                          | 10 weak           | 8 not effective           |
| 45                          | 10 weak           | 8 not effective           |
| 60                          | 12 weak           | 10 weak                   |
| 75                          | 16 medium         | 12 weak                   |
| Decanoic Acid               | 50                | 13 weak                   | 14 weak                   |
| Coconut Oil Fatty Acid      | 50                | 10 weak                   | 10 weak                   |
| Clyndamisin                 | 0.5               | 16 medium                 | -                        |
| DMSO                        | 100               | -                         | -                        |
| Glycerol                    | 100               | -                         | -                        |
| n-hexane                    | 100               | -                         | -                        |
Table 3 Classification of effectiveness of antimicrobial substances

| Inhibit zone diameter | Response of growth barriers |
|-----------------------|-----------------------------|
| >20 mm                | Strong                      |
| 16-20 mm              | Medium                      |
| 10-15 mm              | Weak                        |
| <10 mm                | Not effective               |

The antimicrobial activity of fatty acids is influenced by length of the carbon chain, unsaturation, and location of the unsaturated bond [5]. Short-to-medium chain fatty acids have antimicrobial inhibitory activity especially in the form of monoglycerides, whereas monoglycerides of long-chain saturated fatty acids do not act as antimicrobial agent [17]. The location and number of double bonds on C₁₂-C₂₂ fatty acids also affect microbial activity compared to fatty acids with less than 12 carbon atoms. The addition of cis double bond can increase the antimicrobial activity of a straight chain fatty acid, while the configuration of the fatty acid structure geometry in the trans form is inactive as an antimicrobial agent [17].

Most of the fatty acids in coconut oil are fatty acids with medium carbon chains, i.e., lauric, myristic, and decanoic acid having carbon chains ≤ 16 [2]. This caused the glycerol-coconut oil fatty acid ester produced in this research can act as antimicrobial agent.

4. CONCLUSION

Enzymatic synthesis of glycerol-decanoic and glycerol-coconut oil fatty acid esters using Candida rugosa lipase as biocatalyst have been successfully performed by showing the characteristic -C=O ester group at FT-IR spectra. Glycerol-decanoic and glycerol-coconut oil fatty acid esters have activity as emulsifier for water in oil (w/o) emulsion type. Both ester also showed the antimicrobial activity against Propionibacterium acnes and Staphylococcus epidermidis. Glycerol-decanoic ester shows higher antimicrobial activity than glycerol-coconut oil fatty acid ester.

ACKNOWLEDGEMENT

This work was funded by Hibah Kompetensi Publikasi Internasional Terindeks Untuk Tugas Akhir Mahasiswa (PITTA), Universitas Indonesia, Contract No. 1821/UN2.R3.1/PPM.00.01/2017.

REFERENCES

[1] Manisha, D.M. dan Shyamapada M. 2011. Coconut (Cocos nucifera L. Areaceae): In Health Promotion and Disease Prevention APJTM, 4 (3), 241-247.
[2] Handayani, S; I. Novianingsih; A. Barkah; S. Hudiyono. 2012. Enzymatic synthesis of sucrose polyester as food emulsifier compound. Makara Journal of Science, 141-148.
[3] Zhao, Lel, et al. 2015. In Vitro Antibakterial Activities and Mechanism of Sugar Fatty Acid Esters Against Food-Related BacterJia. Journal Of Food Chemistry 187: 370-37.
[4] Ketaren, S. 1986. Introduction of Oil Technology and Food Fat. Penerbit UI Press. Jakarta.
[5] Kabara JJ; Swiecikowski DM., Conley AJ., Truant JP. 1972. Fatty Acids and Derivatives as Antimicrobial Agents. Antimicrob Agents Chemother 2(1):23–28.
[6] Hudiyono, S.; Risang Guritno; Rahayu Amanda; Sri Handayani; Ridla Bakri. 2015. Immobilized Lipase Candida Rugosa Ec 3.1.1.3 On Nanoparticles Fe₂O₃-Polydopamine As Catalyst Of Esterification Reaction Among Glucose To Palm Oil Fatty Acids. Conference Proceeding of The
3rd Asia-Pacific Conference on Life Science and Engineering (APCLSE). Chiang Mai, Thailand 18-20 November 2015.

[7] Anlisa, S.; Sri Handayani and Sumi Hudiyono. 2015. Effect of Solvents on Palm Oil Transesterification using Immobilized Candida rugosa Lipase on Fe₃O₄-Polydopamine Nanoparticles. International Symposium on Current Progress on Mathematics and Sience (ISCPMS) 2015. Depok, Indonesia 4-5 November 2015. AIP Conf. Proc. 1729, 020044 (2016); http://dx.doi.org/10.1063/1.4946947.

[8] Kautsari, S.N.; Sri Handayani; Sumi Hudiyono. 2015. Interesterification of Palm Oil by using Immobilized Lipase Candida rugosa on Fe₃O₄-Polydopamine Nanoparticles and Isooctane Solvent. International Symposium on Current Progress on Mathematics and Sience (ISCPMS), Depok, Indonesia 4-5 November 2015. AIP Conf. Proc. 1729, 020044 (2016); http://dx.doi.org/10.1063/1.4946947

[9] Adamopoulos, Lambrini 2006 Understanding The Formation of Sugar Fatty Acid Esters. Faculty of North Carolina State University.

[10] Zaks, Aleksey; Klibanov, Alexander. 1985. Enzyme-catalyzed processes in organic solvents. Vol. 82, pp. 3192-3196. USA

[11] Öztürk, Banu, 2001. Immobilization of Lipase from Candida rugosa on Hydrophobic and Hydrophilic Supports. ozmir Institute of Technology, Turkey.

[12] Zaks, A. and A.M. Klibanov. 1988. The Effect of Water on Enzyme Action in Organic Media. J. Biol.

[13] Yoo, I.S., S.J. Park and H.H. Yoon, 2007. Enzymatic Synthesis of Sugar Fatty Acid Esters. J. Ind. Eng. Chem., 13(1): 1-6.

[14] Luna, Prima; Andarwulan, Nuri. 2013. Potensi Produk Monoasilgliserol sebagai Emulsifier Nabati.Buletin Teknologi Pascapanen Pertanian Vol 9 (2), 2013 : 108-116.

[15] Christensen, Clemen. 2011. Emulsifiers used in food applications, focusing on the meat processing industry. Palsgaard Technical Paper.13.

[16] Greenwood. 1995. Antibiotic susceptibility (sensitivity) test, antimicrobial and chemotherapy. USA: Mc Graw Hill Company.

[17] Kabara JJ 1983 Medium-Chain Fatty Acids and Esters. Di dalam: Antimicrobials in Foods. Alfred L. B. dan P.M Davidson (eds). Merceil Dekker Inc., New York and Basel.