

# Synthesis of Glycerol — Castor Oil Fatty Acid and Glycerol — Oleic Acid Esters, as Emulsifier and Antibacterial Agent, Using *Candida rugosa* Lipase

Elmira Vania Denada<sup>1</sup>, Sri Handayani<sup>1</sup> Sumi Hudiyono<sup>1</sup> and Siswati Setiasih<sup>1</sup>

<sup>1</sup>Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, West Java, Indonesia

**Keywords:** Castor Oil, Glycerol, castor oil fatty acid, ester, oleic, emulsifier, antibacterial, *Candida rugosa*

**Abstract:** The research's objective is to synthesize glycerol – castor oil fatty acid and glycerol oleic acid esters using *Candida rugosa* lipase in n-hexane. Molar fatty acid to glycerol ratio that used in esterification varied from 1:1, 1:2, 1:3, and 1:4. The ester products then being analysed by using FTIR. The spectra for glycerol-castor oil fatty acid ester and glycerol-oleic acid absorption peak at wave number 1735.37 cm<sup>-1</sup> and 1748.25 cm<sup>-1</sup> respectively, which indicate the existence of C=O ester groups. Conversion percentage in esterification was conducted using titrimetric method. The highest conversion percentage was reached at the molar ratio 1:4 with the value of 92.4% respectively for glycerol-castor oil fatty acid and 86.2% for glycerol oleic ester. Emulsifier test was performed to observe the stability and the type of emulsion, using ester product as emulsifier. The properties of the emulsifier in both esters are obtained by type of water in oil. Antimicrobial assay were also conducted for both esterification product using disc diffusion method against *Propionibacterium acne* and *Staphylococcus epidermidis*. The antimicrobial assay showed that glycerol-castor oil fatty acid esters has antimicrobial activity, but glycerol-oleic esters did not show the antimicrobial activity.

## 1 INTRODUCTION

Castor oil from castor bean plant has many benefits, such as biodiesel source, plantation commodities, pharmacology, natural insecticides, and also as basic materials for moisturizers and cosmetics (Alvarez and Rodriguez, 2000; Amir and Hartono, 2013; Santoso et al, 2014). Generally, 89% from all of fatty acid in castor oil is ricinoleic (Omari et al, 2015). Ricinoleic can be obtained by hydrolysis of castor oil which involves high pressure and temperature in the presence of catalyst (Yamamoto and Fujiwara, 1995). This reaction will produce glycerol and ricinoleic fatty acid.

Ricinoleic fatty acid from castor oil may gain higher economic value by converting them into lipid derivate compounds. Lipids in the form of monoglycerides or diglycerides are often used as emulsifiers for cosmetics. Fatty acid monoesters are one of the compounds from lipid derivatives, which have the highest antibacterial activity against gram positive and gram negative bacteria (Zhao et al, 2015).

The esterification reaction between fatty acid and glycerol is capable of producing glycerol derivative compounds such as monoglycerides, diglycerides, and triglycerides. The glycerol monoester compound has significant applications in food, pharmaceutical, cosmetics, and even detergent industries (Pouilloux et al, 2000). Mono- and diglycerides may also serve as emulsifiers. In addition, Kosová, et al (2015) and Handayani, et al (2018), stated that fatty acid glycerol ester has antimicrobial activity. In conventional chemical methods, esterification processes often involve acid catalysts. However, this method provides an unwanted by-product (Zaidi et al 1995), so that esterification using enzymes – such as lipase – is more commonly used in modern chemical methods.

Lipase is a hydrolase enzyme. *Candida rugosa* lipase often used as catalyst in the reaction of triglyceride hydrolysis. *Candida rugosa* lipase can also be used as a catalyst in esterification reactions. However, it should be in a certain condition, namely the small amount of water and the use of organic solvents (Zaks and Klibanov, 1984).

## 2 MATERIALS AND METHODS

### 2.1 Materials

Aquades, *Candida rugosa* lipase (2.45 U/mg) from Sigma-Aldrich, 96% ethanol, castor oil, commercial glycerol, Clindamycin, DMSO 10%, eosin, hydrogen chloride, n-hexane, nutrient broth, nutrient agar, oleic acid, phenolphthalein, phosphate buffer pH 8, potassium hydroxide, sodium hydroxide, *Propionibacterium acnes* and *Staphylococcus epidermidis* bacteria, are materials used in this study.

### 2.2 Methods

#### 2.2.1 Hydrolysis of Castor Oil

Castor oil hydrolysis conducted by mixing 100 grams of the compound with 100 mL KOH 5 M in ethanol, heated and stirred using hotplate for 2 hours at  $70 \pm 2^\circ\text{C}$ . The mixture were then allowed to reach room temperature and added by 50 mL HCl 5 M while stirring for two hours. The castor oil fatty acid was obtained by let the mixture stand for 24 hours until two phased formed. The upper phase were taken as castor oil-fatty acid.

#### 2.2.2 Esterification Reaction

Esterification delivered by mixing fatty-acid, glycerol, n-hexane (as a solvent) and *Candida rugosa* lipase as biocatalyst. Solvent to substrate ratio was 1:1 (v:v) while fatty acid to glycerol ratio were varied among 1:1, 1:2, 1:3, and 1:4 (mol:mol) and for enzyme use was 5% of the substrate's total mass. Horizontal incubator was used to incubate the mixture. The incubation process was carried out on 18 hours at 200 rpm and  $37^\circ\text{C}$ . Termination for the esterification reaction obtained by heating the mixture at  $\pm 80^\circ\text{C}$  to denaturate the enzyme. To separate the mixture, centrifugation process was executed at 3400 rpm in 15 minutes. Each of the layers formed were separated and the middle one was taken as an ester product.

#### 2.2.3 Conversion Percentage Determination

The percentage of conversion determined by titrimetric method. 0.1 N NaOH employed to titrate the hexane phase (upper-phase), and phenolphthalein was involved as an indicator.

#### 2.2.4 Ester Product Characterization using FTIR

FTIR was used to characterized the ester product, castor oil, oleic acid, and castor oil-fatty acid.

#### 2.2.5 Emulsifier Test

Oil and water mixed with 0.1 g ester products to test the emulsion properties. The oil and water amounts shown on Table 1. The mixture were shaken using vortex for 30 seconds and the stability of the emulsion was observed.

Table 1. Oil and Water Amount

	Water (mL)	Oil (drops)
Variation One	2	10
	Water (drops)	Oil (mL)
Variation Two	10	2

#### 2.2.6 Emulsion Type Determination

A drop of emulsion and eosin respectively were mixed on an object glass. Then the mixture was observed under the microscope to define the type of emulsion, whether oil in water (o/w) or water in oil (w/o).

#### 2.2.7 Antimicrobial Activity Assay

Clindamycin which used as positive control are involved in Antimicrobial activity assay. The activity was performed with disc diffusion method towards castor oil, castor oil-fatty acid, ester products, glycerol, n-hexane and DMSO as solvent, and oleic acid. In a sterile petri dish, 20 mL nutrient agar mixed aseptically with  $2 \times 10^2$   $\mu\text{L}$  aliquot of *Propionibacterium acnes* suspension which its cell density is  $1 \times 10^8$  cells/mL. The medium were then allowed to harden. Then, the top of the medium placed by paper disc and  $400 \times 10^{-2}$   $\mu\text{L}$  sample was dropped to it. Incubation for the medium was held at  $37^\circ\text{C}$  for 24 hours. The microbial activity calculated based on the diameter of the clear zone on the medium. This assay were also done for *Staphylococcus epidermidis*.

### 3. RESULT AND DISCUSSION

#### 3.1 Castor Oil Hydrolysis

The catalyst used for hydrolysis reaction was potassium hydroxide. Ethanol, as a semi polar compound, used for this reaction to be an intermediary of potassium hydroxide and triglycerides which leads the reaction to be occurred. The mixture was then added by HCl to acidify and form the fatty acid. Yield percentage obtained was 87.5%.

#### 3.2 Esterification

This reaction was catalysed by *Candida rugosa* lipase in organic solvent with small amount of water. Although *Candida rugosa* as hydrolase enzyme, it is often used as a catalyst in hydrolysis reaction. In this case it can also be used as a catalyst in the esterification reaction with requirement of organic solvent and small amounts of water (Zaks and Klibanov, 1984). In this study, n-hexane was used as solvent in the enzymatic esterification. n-hexane, the non-polar compound, is capable to dissolve fatty acid. Phosphate buffer pH 8 was used to optimize *Candida rugosa* lipase and dissolve glycerol. *Candida rugosa* lipase has a low activity when the pH used is less than 6.0 or more than pH 8.0 (Öztürk, 2001).

The molar ratio variation of fatty acid:glycerol were intended to observed the optimum formation of ester and also to keep the reaction equilibrium in the direction of the ester. The highest ester product produced by using molar ratio 1:4. The ester formed supposed to be mono- or diglycerides since the amount of fatty acid used was lower than the glycerol.

#### 3.3 Conversion Percentage Determination

The three layers from centrifugation process on esterification were separated and the top layer was used to determine the conversion percentage.

Figure 1 describe the conversion percentage from all of variations (mol:mol). The highest percentage was reached at the molar ratio 1:4 (fatty acid:glycerol) with value of 92.4% for ester glycerol-castor oil fatty acid and 86.2% for ester glycerol oleic acid.

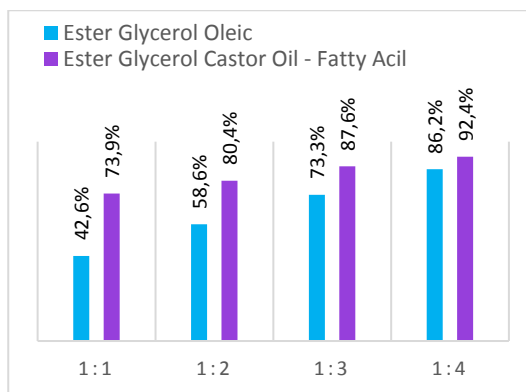
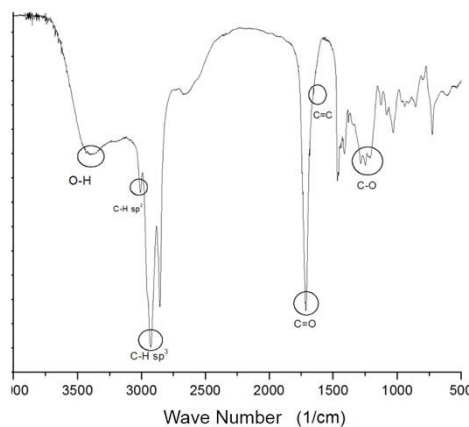
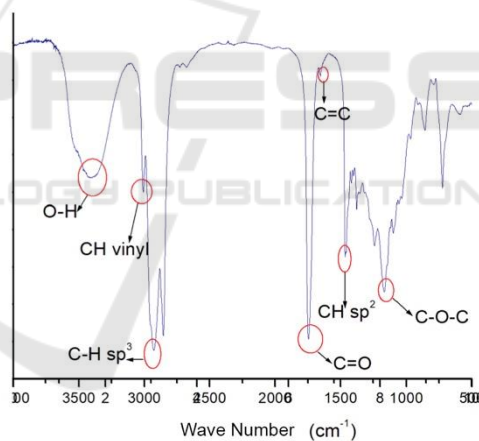


Figure 1. The Curve of Variation of Mol vs Percentage of Conversion

#### 3.4 Ester Products Characterization by FTIR

Figure 2 and Figure 3 below show the IR spectrum of castor oil, castor oil-fatty acid, oleic acid and ester products.



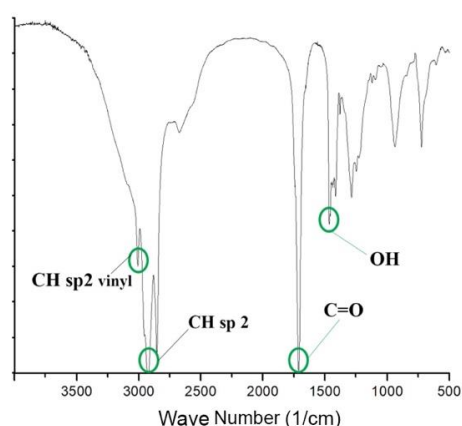


Figure 2. FTIR Spectrum of a) Castor Oil, b) Castor Oil-fatty acid, c) Oleic Aid

FTIR spectrum above shows the presence of CH  $sp^2$  vinyl group which indicates the presence of double bonds in castor oil and castor oil-fatty acid hydrolysis. In addition, there is also a C=O functional group detected in castor oil, but its wave number shifts when the castor oil has been hydrolysed to fatty acids. The C=O functional group in castor oil is identified as C=O carboxylate at wave number  $712.97\text{ cm}^{-1}$  (Figure 2a). Then on the castor oil-fatty acid, the functional group identified as C=O ester at wave number  $1745.65\text{ cm}^{-1}$  (Figure 2b). Oleic acid has a typical functional group, specifically C=O carboxylate ( $714.61\text{ cm}^{-1}$ ) and CH  $sp^2$  vinyl ( $3009.97\text{ cm}^{-1}$ ) (Figure 2c). CH  $sp^2$  vinyl group indicates the presence of double bond of the compound. As well known, oleic acid has a double bond on C<sub>9</sub> and C<sub>10</sub>.

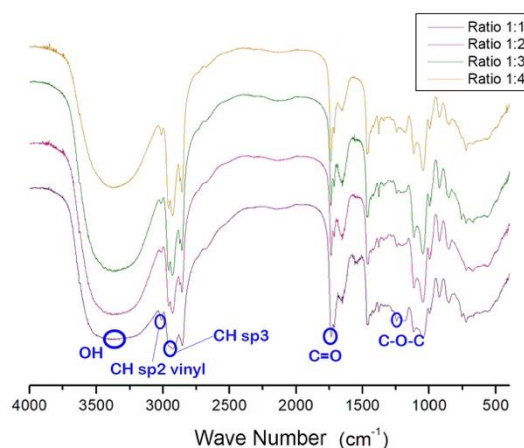


Figure 3. a) Ester Glycerol Oleic, b) Ester Glycerol-Castor Oil Fatty Acid

Figure 3a. proves that oleic acid has been esterified, since the spectrum states the presence of C=O ester groups at wave number  $1748.25\text{ cm}^{-1}$ . In addition, there are also OH groups ( $3319.63\text{ cm}^{-1}$ ) whose peaks look widened. The presence of a widened OH group caused by the hydroxyl group present in glycerol is not fully esterified. Furthermore, the absorption band of the carboxylate group is still visible, so it can be said the ester glycerol oleic still contain oleic acid.

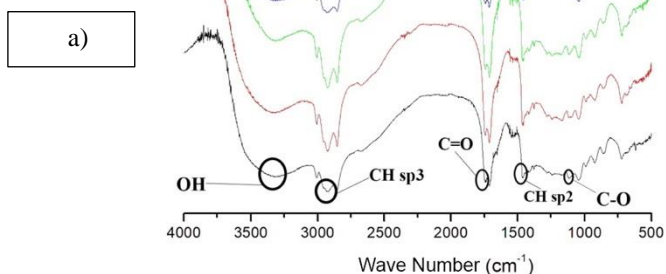
Figure 3b. shows the absorption band of the OH functional group of the compound ( $3410.07\text{ cm}^{-1}$ ). The carbon chain of the ester compound is shown by the functional group CH  $sp^3$  ( $2923.53\text{ cm}^{-1}$ ). The identical group of esters, obtained as the C=O ester group ( $1735.37\text{ cm}^{-1}$ ) has been shown. It can be said that the castor oil-fatty acid results have been esterified.

Those functional groups as existed in Silverstein et al (1981), the spectrum of CH  $sp^2$  vinyl is in the range  $3010 - 3100\text{ cm}^{-1}$ , whereas C=O ester is in the range  $1735 - 1750\text{ cm}^{-1}$  and C=O carboxylic acid at  $1700-1725\text{ cm}^{-1}$ , also OH is at  $3200 - 3400\text{ cm}^{-1}$ , and CH  $sp^3$  at  $2850 - 3000\text{ cm}^{-1}$ .

### 3.5 Emulsifier Test

Result of the emulsifier test indicates that the ester glycerol oleic and ester glycerol-castor oil fatty acid have emulsifier properties.

The emulsifier test showed that ester glycerol-castor oil fatty acid and ester glycerol oleic have emulsifier properties. The emulsion of ester glycerol-castor oil fatty acid more stable than the emulsion of ester glycerol oleic (Figure 4). The emulsions stable up to 36 hours for ester glycerol-castor oil fatty acid and 24 hours for ester glycerol oleic.





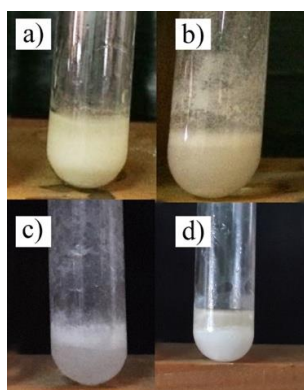


Figure 4. Emulsion test results; a) Variation Two of Ester Glycerol-Castor Oil Fatty acid, b) Variation One of Ester Glycerol-Castor Oil Fatty Acid, c) Variation One of Ester Glycerol-Oleic, d) Variation Two of Ester Glycerol-Oleic.

### 3.6 Emulsifier Type Determination

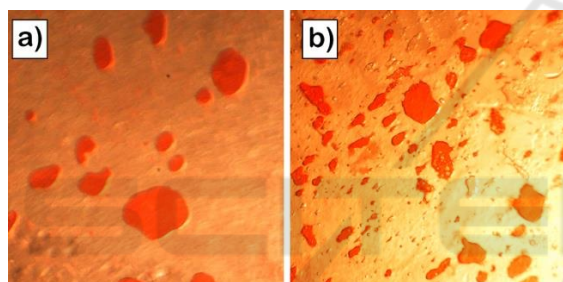


Figure 5. Emulsion type of; a) Ester Glycerol-Castor Oil Fatty Acid, b) Ester Glycerol Oleic

Emulsion's type was determined through observation under a microscope. Eosin was added as a water-soluble dye because of its polar nature. So that the water phase will change colour to red. The presence of eosin makes it easy to observe the type of emulsion because the water phase and the oil phase will have different colours.

The ester glycerol casto oil-fatty acid and ester glycerol oleic have a water-in-oil emulsion type (w/o) (Figure. 5). The red droplets are water, and the yellow environment is oil.

### 3.7 Antibacterial Activity Assay

*Propionibacterium acnes* and *Staphylococcus epidermidis* were used in this study. Ester glycerol-castor oil fatty acid as well as ester glycerol oleic, were varied in 20%, 40%, 60%, and 80% (w/w). This variation were used to determine the best concentration of esters that can inhibit bacterial growth. Table 2 shows the classification of antimicrobial effectiveness substances (Greenwood, 1995). Table 3 shows the antimicrobial activity.

Table 2. Classification of Antimicrobial Effectiveness

Inhibition Zone Diameter	Response of Growth Barriers
> 20 mm	Strong
16 – 19 mm	Average
10 – 15 mm	Poor
< 10 mm	Ineffective

Table 3. Inhibition Zone of Various Compounds in Antibacterial Activity Assay

Sample	Inhibition Zone Diameter (mm)		Classification
	<i>P. acnes</i>	<i>S. epidermidis</i>	
Ester Glycerol-Castor Oil Fatty Acid 20%	-	-	No Activity
Ester Glycerol-Castor Oil Fatty Acid 40%	7	9	Ineffective
Ester Glycerol-Castor Oil Fatty Acid 60%	9	9	Ineffective
Ester Glycerol-Castor Oil Fatty Acid 80%	12	10	Poor
Ester Glycerol Oleic 20%	-	-	No Activity
Ester Glycerol Oleic 40%	-	-	No Activity
Ester Glycerol Oleic 60%	-	-	No Activity
Ester Glycerol Oleic 80%	-	-	No Activity
Castor Oil Fatty Acid 50%	12	14	Poor
Castor Oil Fatty Acid 100%	13	15	Poor
Oleic Fatty Acid	-	-	No Activity
Castor Oil 100%	-	-	No Activity
n-hexane 100%	-	-	No Activity
Glycerol 100%	-	-	No Activity
DMSO 100%	-	-	No Activity
Clindamycyn 0.5%	18	15	Average

The data above shows that there was no antimicrobial activity from oleic acid and esters glycerol oleic against *Staphylococcus epidermidis* and *Propionibacterium acnes*. The absence of antimicrobial activity also found in glycerol, n-hexane, and DMSO. It clearly shows that the solvent used in this study has no antimicrobial activity.

In general, the increases of concentration in test compounds were able to produce larger inhibition zone diameters for both types of bacteria (Table 3). Ester glycerol-castor oil fatty acid has the highest value at 80% concentration by producing 12 mm inhibition zone for *Propionibacterium acnes* bacteria. In the same concentration, the ester is capable of providing 10 mm inhibition zone for *Staphylococcus epidermidis* bacteria. Thus, it can be said that ester glycerol-castor oil fatty acid has a stronger inhibitory effect against *P. acnes*.

The inhibition zone can also be found in castor oil-fatty acid. The highest diameter at 100% concentration with 13 mm and 15 mm, respectively for *P. acnes* and *S. epidermidis*. Castor oil fatty acid and ester glycerol-castor oil fatty acid are in the same classification of inhibitory resistance, which is poor. Inhibition zones of clindamycin are 18 mm for *P. acnes* and 15 mm for *S. epidermidis*. This researched showed that ester glycerol-castor oil fatty acid have the activity for antimicrobial agent.

## 4 CONCLUSIONS

Glycerol – castor oil fatty acid and glycerol – oleic esters were successfully synthesized using *Candida rugosa* lipase as catalyst. Both ester products can be used as emulsifier for water in oil (w/o) emulsion type. Only glycerol-castor oil fatty acid ester has antimicrobial activity.

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