Enzymatic Esterification Ethyl Ester Fatty Acid from Hydrolyzed Castor Oil and its Oxidation Product as Emulsifier and Antimicrobial Compound Using Candida rugosa Lipase E.C.3.1.1.3

Annisa Khairani[1], Sumi Hudiyono[1] and Sri Handayani[1]
[1]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, West Java, Indonesia*

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Abstract: Aim of this study was to synthesis fatty acid ethyl ester compound of hydrolyzed castor oil and its oxidation product using *Candida rugosa* lipase. Both of the esterification products were expected to have antimicrobial activity against *Staphylococcus epidermidis* and *Propionibacterium acnes* and may act as emulsifiers. Optimization of esterification reactions was done by varying the mole ratio between fatty acids and ethanol, ie 1:1, 1:2, 1:3, and 1:4. The esterification products were then characterized using FTIR. Conversion percentage was determined by titrimetric method, to calculate the amount of fatty acids that was converted to ester. The ester product was tested its ability as emulsifier by emulsifier test. The esterification products were also examined their antimicrobial activity using disc diffusion method. The highest conversion percentage for fatty acid ethyl ester and its oxidation product were 76 % and 72% respectively. Characterization using FTIR for both ester showed that the absorption band of C=O ester functional group at wave number 1731.27 cm^{-1} and 1732.15 cm^{-1} respectively. The emulsifier test showed that both esters have ability to stabilize emulsion up to 24 hours for water-in-oil emulsion (w/o) type. Antimicrobial assay showed that both esters have antimicrobial activity against both bacteria.

1 INTRODUCTION

Indonesia is a tropical country that presents a wide range of beneficial biological plants that can grow in it. One of the oil-producing plants that benefit the wider community is *Ricinus communis* L. *Ricinus communis* L. is an oil-producing plant (castor oil) which contains triglyceride from a variety of fatty acids which largest content is risinoleic acid, i.e 85-95% of the total fatty acids (Gunstone et al.,2007). Risinoleic acid is a long-chain fatty acid having 18 carbons which the ester form is known to have strong antibacterial activity against Gram-positive bacteria (Desbois and Lawlor, 2013) and used as emulsifier in cosmetic industry (Cavalcante et al., 2009).

Ricinoleic acid can be obtained by castor oil hydrolysis in an alkaline solution. Ricinoleic acid can also be esterified with ethanol (Hykkerud and Marchetti, 2016). To achieve the equilibrium of an esterification reaction that is so slow, catalyst is required to accelerate it. Lipase was used in enzymatic esterification using ethylene glycol at low temperature and pressure (Chand et al., 1997).

In this research, the esterification between ethanol and fatty acid obtained from the hydrolysis of castor oil used *Candida rugosa* Lipase as catalyst. Esterification also conducted between ethanol and oxidized castor oil fatty acid. The ester products were then examined as emulsifier and antibacterial compound.

2 MATERIALS AND METHODS

2.1 Chemicals

The materials used in this study were ethanol, KOH, ethanol 96%, concentrated HCl, NaOH 0.5 N, aquades, Na_2SO_4 anhydrous, n-hexane, phosphate buffer pH 8, KI 15%, thiosulfate 0.01 N, amilum 1 %, $KMnO_4$ 1M, phenolphthalein indicator, eosin indicator, clindamycin antibiotics, sterile aquades, DMSO, nutrient broth and nutrient agar,

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Staphylococcus epidermidis and *Propionibacterium acnes* cultures (obtained from Biochemistry Lab Department of Chemistry, Universitas Indonesia). *Candida rugosa* Lipase obtained from Sigma-Aldrich.

2.2 Hydrolysis of Castor Oil

To get hydrolized castor oil fatty acid 100 g of castor oil and 100ml KOH 5 M solution in 96% were mixed. The mixture was heated in oil bath for 1 hour at 70 ^{o}C with magnetic stirrer and was cooled at room temperature. HCl 5M was added until pH 5-4 (± 55 mL). The mixture was then allowed to stand for 24 hours and will formed 2 phases. The upper phase (organic phase) were separated and called hydrolized fatty acids.

2.2.1 Oxidation of Fatty Acids

To 10 ml of hidrolyzed fatty acid 5 mL NaOH 0.5 M and 2 mL of $KMnO_4$ was added and stir for 90 min at 25 ^{o}C. The solution was then left for 24 hours, then filtered. The filtrate was added by 4 mL of sulfuric acid. The Na_2SO_4 anhydrate was added to the organic phase of oxidized sample, and decanted.

2.2.2 Iod Numbers Test

Before and after oxidized, 0.3-0.4 g fatty acid was mixed with 10 mL of chloroform and 10 mL of Hanus solution. The solution was stored for 30 minutes in dark place. Furthermore, 10 mL of 15% KI solution and 100 mL of aquadest were added. The solution was titrated using 0.1 N sodium sulfate solution to a yellow colour. The solution was added 1-2 mL of 1 % amilum solution and re-titrated with 0.1 N sodium sulfate solution until the colour turned clear (Goud, 2006).

2.3 Esterification

2.3.1 Synthesis of Ethyl Ester Hydrolyzed Castor Oil Fatty Acid and Its Oxidation Products Using *Candida Rugosa* Lipase

To get ester products fatty acid and the n-hexane, *Candida rugosa* lipase as catalyst and ethanol were mixed. Before mixing *Candida rugosa* was dissolved with pH 8 phosphate buffer solution. The mol ratio of fatty acid to etanol (respectively) varians were 1: 1, 1: 2, 1: 3 , and 1: 4 (mol/mol). The amount of solvent were used are 1:1 (v/v substrate in each ratio). The 5% of the substrate total mass was used as enzyme mass (w/w of each substrate ratio). The incubation was conducted using horizontal incubator shaker at 250 rpm and 37 °C for 18 hours. To terminate the reaction, the mixture was heated in a water bath at temperature of ± 80 ^{o}C for 1-3 minutes. The same treatment was applied for oxidized fatty acid ethyl ester.

2.3.2 Conversion Percentage Determination

Conversion percentage was calculated by using titration method. Titrations were performed on an organic phase (upper phase) which is a residual fatty acid dissolved in n-hexane. 1 mL the organic phase that has been separated after the centrifugation process is transferred into a 10 mL measuring flask and adjusted its volume with n-hexane. Then as much as 1 mL aliquot was titrated with 0.1 N NaOH.

2.3.3 Identification Product Using FTIR

Esterification products, hydrolyzed castor oil fatty acid, and oxidized fatty acids were identified using FTIR.

2.4 Emulsifier Test and Determination of Emulsion Type

Emulsifier test was carried by mixing water and oil with a certain ratio according to Table 1. A total of 0.1 g of fatty acid ester were added into each mixture, then shaken using a vortex for 30 seconds to form an emulsion. Then the stability emulsion was observed.

Table 1 : Oil and water composition for emulsions

Emulsion Type	Water (ml)					Oil (drop)				
Type 1	1	1	1	1	1	2	4	6	8	10
	Water(drop)					Oil (ml)				
Type 2	2	4	6	8	10	1	1	1	1	1

To determine the emulsion type, a drop of emulsion and eosin was mixed on object glass. The observation of emulsion type was performed under microscope to determine an oil-in-water (o / w) oil or water in oil (w / o) emulsion type.

2.5 Antimicrobial Activity Assay

Disc diffusion method was used as antimicrobial activity assay. Aliquot 200 µL of *P.acnes* and *S.epidirmidis* suspension with cell density $1x10^8$

cells/mL was mixed with 20 mL nutriet agar in sterile petri dish aseptically. The media was then allowed to harden. Sterile disc paper with diameter of 6 mm was placed on top of the medium and dropped by 4 µL on sample. The incubation was performed at 37 °C for 24 hours. Clindamycin (0.5%) was used as positive control while DMSO as negative control. The clear area around disc paper was measured to determine antimicrobial activity. The assay was performed on ethyl ester fatty acid product with concentrations of 20%, 40%, 60%, and 80%.

3 RESULT AND DISCUSSION

3.1 Hydrolysis of Castor Oil

The hydrolysis process in this study was carried out using an alkaline as catalyst in order to obtain fatty acids from castor oil. The hydrolysis reaction with an alkaline catalyst is irreversible, thereby resulting in a higher fatty acid yield than the reversible acid catalyst (Rifqy, 2016). To obtain free fatty acids, the resulting soap was added by HCl. The fatty acid obtained from this process was about 83.5%. The hidrolized castor oil fatty acid was identified using FTIR and the spectrum can be seen in Figure 1.

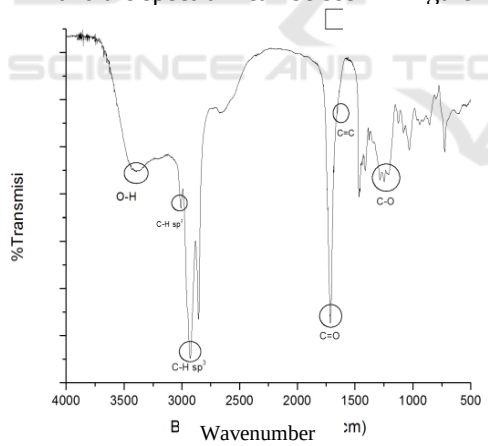


Figure 1: Castor oil hydrolyzed fatty acid spectrum.

The figure showed absorbsion at wave numbers 1725-1700 cm^{-1} which indicate C=O carbonyl uptake bands of carboxylic groups after hydrolysis process. At wave number 1800-1650 cm^{-1} appears the absorption band for C=C accompanied by the appearance of absorption band CH sp^2 vinyl at wave number 3150-3000 cm^{-1} (Janice, 2011).

3.3.1 Oxidation of Fatty Acids

The fatty acids obtained from castor oil hydrolized have a double bond (C=C) which may undergo a oxidation reaction. The oxidation process was performed to increase the hydroxyl group into the molecule by breaking the double bond (Pierre, 1994). The oxidation reaction using $KMnO_4$ at an alkaline condition provides the oxidation product of a diol compound (Marlina, 2004). The spectrum of oxidized can be seen in Figure 2.

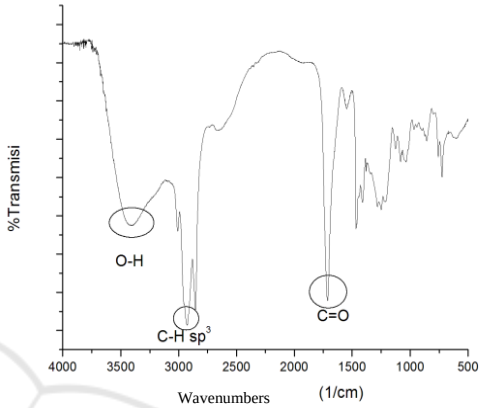


Figure 2: Oxidized fatty acid spectrum.

The loss of the absorption band for C=C and the CH sp^2 absorption bands indicated that the fatty acids had been oxidized to diols . The presence of hydroxyl group of diols were shown with sharper and wider absorbing bands for O-H.

The determination of the iodine is performed to prove that the fatty acid had been oxidized. In this study showed that iodine number on hydrolyzed fatty acid is 10 mg/g while the oxidized fatty acids of 1.5 mg/g. It indicated that the oil has lost its C=C and the oxidation was successful.

3.2 Esterification

Candida rugosa Lipase can catalyze the esterification reaction under a slight water condition, called essential water. Enzyme need water to perform its catalytic activity called essential water (Zaks and Klibanov, 1988). The essential water in this study was obtained from pH 8 phosphate buffer. The use of alcohol in larger quantities may induce a reaction towards ester formation (Pandey et al., 1999). In order to optimize the esterification reaction, the reaction equilibrium is shifted to the right direction to the formation of ester. In this research, ethanol was used in excess amount. The ester was identified using FTIR and the spectrum can be seen in Figure 3.

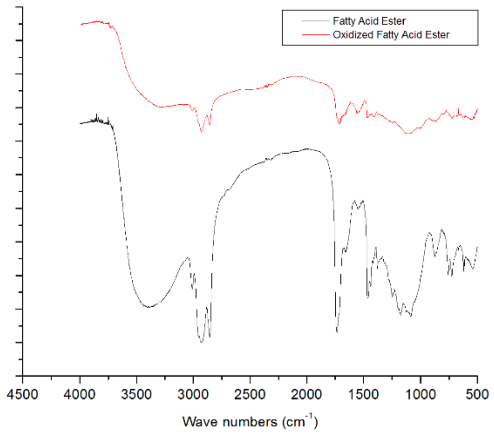


Figure 3 : Esters spectrum.

The figure shows the presence of the C = O ester absorption bands at the wave numbers 1735-1750 cm^{-1} for both types of esters. This bands shows a shift to greater wave numbers than fatty acid and oxidized fatty acid form, this wave number is also the typical wave number for C=O groups of ester. The differences between these two spectrum are the absence of C=C and C-H vinyl absorbtion bands for oxidized fatty acid esters that appear at wave numbers of 1652.48 cm^{-1} and 3009.73 cm^{-1} (Janice, 2011).

3.2.1 Conversion Percentage

For the castor oil hydrolyzed fatty acids has the highest convertion value at 1:3 composition with conversion percentage value of 76.31% and the oxidized fatty acid has the highest conversion percentage value at 1:2 composition with the value of 72% (Figure 4).

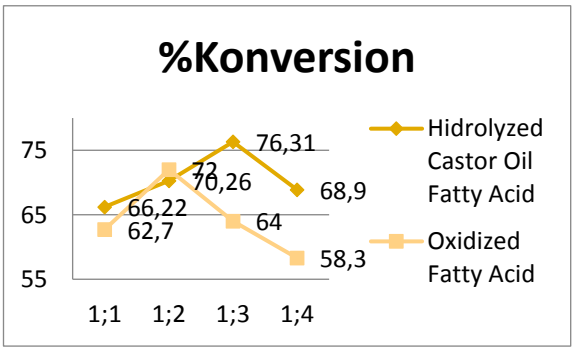
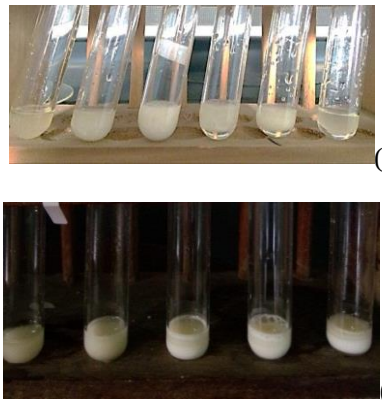


Figure 4 : Konversion percentage ester fatty acid

3.3 Emulsifier Test

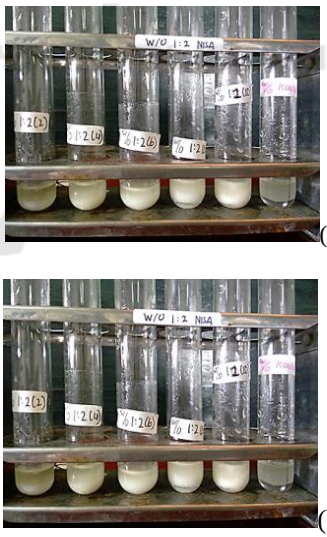
The ester product has a polar group on one side and a non-polar group on the other side, allowing the ester to have properties as an emulsifier. Emulsifier test for castor oil hydrolyzed fatty acid did not produce emulsifier properties, whereas for its oxidation product it gives properties as emulsifier after 24h. This emulsifiers can be seen in Figure 5.


(a)

(b)

Figure 5 : Emulsifiers, (a) Castor Oil Hidrolized Fatty Acid ; (b) oxidized fatty acid

In this study, these two fatty acid esters provide properties as emulsifiers but for the oxidized fatty acid ethyl ester provide more stable emulsion for 48h. This emulsifier test can be seen in Figure 6.


(a)

(b)

Figure 6 : Emulsifiers ester, (a) castor oil hidrolized fatty acid ; (b) oxidized fatty acid

This indicated that ester of oxidized fatty acid has stronger ability as emulsifier, due to addition of hydroxyl groups to the fatty acid structure that increase its polarity. The increasing polarity is able to reduce the surface tension between 2 types of

polar and non-polar solutions (in this case water and oil) (Arbianti, et al., 2009).

The type of emulsion was determined qualitatively under microscope observation. Figure 7 shows microscopic photograph of emulsion. The red part is eosin dissolved in water while the yellow part is the oil phase. In this study, the esters has water in oil emulsion type.

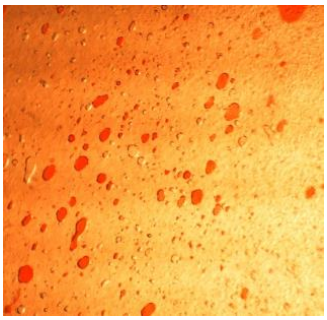


Figure 7 : The observed emulsion type with microscope

3.4 Antimicrobial Activity Assay

Antimicrobial activity is shown by clear areas around paper disc that indicate the inhibition of microorganism growth by antimicrobial agents (Pratiwi, 2008). The stronger the antibacterial activity of the compound, the greater the diameter of clear zone. Based on this research, it was shown that the oxidized fatty acid, the oxidized fatty acid etyl ester at concentration of 40% and 80% had the highest antibacterial activity. All the data of antibacterial activity assay can be seen in Table 3. Table 2 show effectivity parameters for antimicrobial compound.

Table 2: Effectivity parameters for antimicrobial compound (Greenwood, 1995)

Diameter of clear zone	*Classification*
> 20mm	Strong
16-20 mm	Medium
10-15 mm	Weak
< 10 mm	No Effective

The ability of a monoglyceride as an antimicrobial agent is related to the level of solubility of the compound in water so that it can dissolve in an aqueous environment, and its hydrophobicity properties so as to interact with the cell membrane structure composed of lipid bilayer. The longer the chain of C atoms, the more non polar the compound, so the solubility in the water decreases (Widiyarti et al, 2009). The oxidized fatty acid has a polar group at the diol group when it oxidizes the C=C chain. Oxidized fatty acids and its ester product showed greater activity than its fatty acid form because they have a stronger polar side and a longer non-polar side. The antimicrobial activity assay can be seen in Figure 8.

(a) (b)

Figure 9 : Antimicrobial, (a) *Staphylococcus epidermidis* ; (b) *Propionibacterium acnes*

Table 3 : Inhibition Zone

Sample	Inhibition Zone (mm)		Classification
	P. acnes	*S. epidirmidis*	
Ethyl Ester Hidrolyzed Castor Oil Fatty Acid (20%)	3	10	Weak
Ethyl Ester Hidrolyzed Castor Oil Fatty Acid (40%)	X	7	No Effective
Ethyl Ester Hidrolyzed Castor Oil Fatty Acid (60%)	X	8	No Effective
Ethyl Ester Hidrolyzed Castor Oil Fatty Acid (80%)	X	X	No Activity
Oxidized Fatty Acid Ethyl Ester (20%)	12	13	Weak
Oxidized Fatty Acid Ethyl Ester (40%)	17	13	Medium
Oxidized Fatty Acid Ethyl Ester (60%)	14	12	Weak
Oxidized Fatty Acid Ethyl Ester (80%)	12	17	Medium
Oxidized Fatty Acid	15	17	Medium

Ethanol	11	14	Weak
Hidrolyzed Castor Oil Fatty Acid (50%)	12	14	Weak
Hidrolyzed Castor Oil Fatty Acid (100%)	13	15	Weak
Klindamisin (500ppm)	11	20	High
N- Heksane	X	X	No Activity
Castor Oil (50%)	X	X	No Activity
Castor Oil (100%)	X	X	No Activity
DMSO	X	X	No Activity

4 CONCLUSIONS

The synthesis of etyl ester castor oil hydrolyzed fatty acid and its oxidation product using *Candida rugosa* lipase was successfully performed. It was shown by the presence of C=O ester group band of FTIR spectra. Etyl ester castor oil hydrolyzed fatty acid and its oxidation product have activity as emulsifier with water in oil emulsion type. Oxidized fatty acid ethyl ester at concentration of 40% has the highest antimicrobial activity against *Propionibacterium acne* bacteria and oxidized fatty acid ethyl ester at 80% has the highest antimicrobial activity againts *Staphylococcus epidermidis* bacteria.

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