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Optimization of Medium-Chain Glycerides Enzymatic Synthesis from Crude Palm Kernel Oil and Their Anti-bacterial Potential

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ABSTRACT

Medium-chain glycerides (MCGs) have been synthesized from crude palm kernel oil (CPKO). CPKO is composed of 77.41% medium-chain fatty acids (MCFA). The synthesis was conducted by reacting CPKO and glycerol, utilizing *Candida Antarctica Immobilized Lipase Enzyme B* (CALB) as a biocatalyst. This study aims to optimize the enzymatic transesterification to produce an optimal yield of MCGs following *Response Surface Methodology* (RSM). The optimal MCGs were obtained at a ratio of substrates of 1:3 mole, CALB load of 0.15% wt, and a temperature of 40°C. The optimal MCGs contain 57.61% MGs and 25.24% DGs. FTIR analysis revealed that MCGs exhibited functional groups of -OH, -CH, -CH_2, -C=O, -CO, and -C-O-C-. Anti-bacterial examination demonstrated that MCGs at a concentration of 4 mg/mL inhibited the growth of gram-negative bacteria *Escherichia coli*, with the highest zone of inhibition 11.2 mm observed after 5 days of incubation. In conclusion, MCG is classified as a water-in-oil emulsifier with a hydrophile lipophile balance (HLB) of 3.55 and exhibits anti-bacterial properties. The application of MCGs can prevent food spoilage by microorganisms and increase the shelf life of food products.

1. INTRODUCTION

Medium-chain glycerides (MCGs) are non-ionic emulsifiers that have both a hydrophilic head group (glycerol) and a lipophilic tail group (fatty acid) in one molecule [1, 2]. MCGs generally contain medium-chain fatty acids (MCFA) consisting of 8-14 carbon atoms [3, 4]. MCGs have specific applications in foods [5-9], pharmacy [10-13], cosmetics [14, 15], and household products [16]. MCGs are mainly a mixture consisting of mono-di-and triglycerides [17]. MCGs are produced using two methods, namely, chemical and enzymatic transesterification. Today, commercial MCGs are produced through chemical transesterification of MCFA with glycerol using a base catalyst (NaOH, KOH) at 200-250°C [18, 19]. There are several disadvantages to chemical transesterification: low reaction selectivity (±30-40%), lengthy purification steps, soap residue from the uncontrolled saponification reaction between fatty acid and base catalyst [20], dark color product; and burning taste [21]. Commercially MCGs contain 45-55% mono-glycerides (MGs), 38-45% di-glycerides (DGs), and 8-12% triglycerides (TGs) and fatty acids [22]. Enzymatic transesterification offers several advantages. It has high reaction selectivity, is an eco-friendly process, is non-toxic, has no soap formation, produces brighter-colored products, improves sensory characteristics [23, 24]. Therefore, enzymatic transesterification is a promising method to produce MCGs and is feasible to develop on an industrial scale [24]. Commercial MCGs are usually produced from one type of MCFA, such as myristic acid, capric acid, and lauric acid. The utilization of one type of MCFA forms only one type of MCGs. Different fatty acids that are used as raw materials result in MCGs with different applications. For example, the majority of mono-laurate is applied in pharmaceuticals [25] and cosmetics [2, 15]. Mono-palmitate and mono-stearate are applied in food products such as oleogel [26], infant formula, and food babies [27]. The more MCFA contained in MCGs, the broader the application of MCGs [5, 22, 28].

Previous studies showed that MCGs synthesis from MCFA such as glycerol mono-caprate, glycerol mono-laurate, glycerol mono-myristate, glycerol mono-palmitate, and glycerol mono-stearate were reported to have anti-microbial activity against pathogenic strains bacteria of *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Escherichia coli* in food products [28]. Another study also reported that glycerol mono-laurate application in broilers improved eggshell quality and inhibited *Euryarchaeota bacteria* and *Proteobacteria* that can damage eggshells [29]. Glycerol mono-laurate is also utilized as a growth inhibitor of foodborne pathogens of *Salmonella typhimurium*, *Listeria*

monocytogenes, and *Candida albicans* [12]. Likewise, glycerol mono-caprylate and mono-caprate are reported to aid the absorption of fat-soluble vitamins in the body [30]. MCGs addition in processed foods not only acts as an emulsifier [14]. It also inhibits the growth of food spoilage bacteria [31].

All the previous studies synthesized MCGs from one type of fatty acid through chemical transesterification. In terms of raw material, fatty acids are expensive and their utilization is competitive with food industries (margarine, shortening) [32, 33], cosmetics (body lotion, beauty soap) [34, 35], and pharmaceuticals [36, 37]. The cost of raw materials is high, while other sources are economical and abundantly available. Although these MCGs still have anti-bacterial properties, high reaction temperatures are known to reduce the functional properties of emulsifiers [19]. The application of MCGs from chemical transesterification in food products is being considered because contain small amounts of toxic residues that are not harmful to health if consumed in the long term [38]. To overcome these problems, many studies are needed to synthesize MCGs from raw materials that contain mixed fatty acids using enzymatic reactions, and then be applied in many food products.

Based on the discussion above, crude palm kernel oil (CPKO) is one of the triglyceride sources that meets these criteria. CPKO is a renewable raw material derived from pressing palm kernels and contains 4 types of MCFA, namely capric acid (2.48%), caprylic acid (2.42%), lauric acid (59.83%), and myristic acid (12.68%) [39]. MCFAs are known to have natural anti-bacterial characteristics. CPKO is a by-product of the palm oil industry. Its characteristics do not meet commercial standards and it is therefore rejected by industries. This causes the price of CPKO lower than fatty acids and other triglycerides such as corn oil, sunflower oil, coconut oil, and crude palm oil [40].

The synthesis of MCGs from CPKO through enzymatic transesterification reaction has not been reported previously. This study aims to obtain enzymatic transesterification reaction conditions that produce optimal MCGs yield by utilizing CPKO and glycerol as substrates and examining the anti-bacterial activity of MCGs. Although enzymatic transesterification involves a longer reaction time than chemical transesterification, the MCGs produced are safer for consumption.

2. MATERIAL AND METHOD

2.1 Materials

The material used in this research was CPKO from an oil palm processing plant in North Sumatra Province. Based on gas chromatography (GC) analysis, CPKO consisted of 77.41% MCFAs. CPKO has free fatty acid (FFA) of 5.46% (AOCS Ca 5a-40-1998), moisture content of 0.15% (AOCS Ca 11-55-1998), and ash content of 0.20% (AOCS Ca-2c-25-1998) [41]. Glycerol from biodiesel production waste was purified from the previous study, and an 83.37% glycerol level was obtained. The biocatalyst used was Immobilized *Candida antarctica* lipase B (CALB) from Novozyme Inc. Other chemical reagents in analytical grade: n-hexane, NaOH, and ethanol 96% (Sigma-Aldrich).

2.2 Enzymatic transesterification procedure

Synthesis of MCGs was carried out in a 500 ml bottomed glass batch reactor, and placed on a JH-3 digital magnetic stirrer hot plate. The reactor was equipped with a reflux condenser to avoid solvent loss, and a thermocouple to maintain temperature during the reaction, as shown in Figure 1. The substrates consist of 50 g of CPKO and glycerol homogenized with 150 ml of n-hexane as a solvent for 30 min. The substrate was heated to reaction temperature and CALB was slowly added with a stirring speed of 400 rpm. Reaction conditions followed the experimental design in Table 1. After the reaction lasted for 480 min, the MCGs were separated from unreacted substrates by decantation. The top layer contains MCGs, separated from CALB using vacuum filtration. Furthermore, the filtrate was evaporated using a Buchi R-210 rotary evaporator at 90℃, 420 mmHg to separate n-hexane from MCGs. Yield of MCGs determined by GC analysis (MPOB-P3-4-2004) [42].

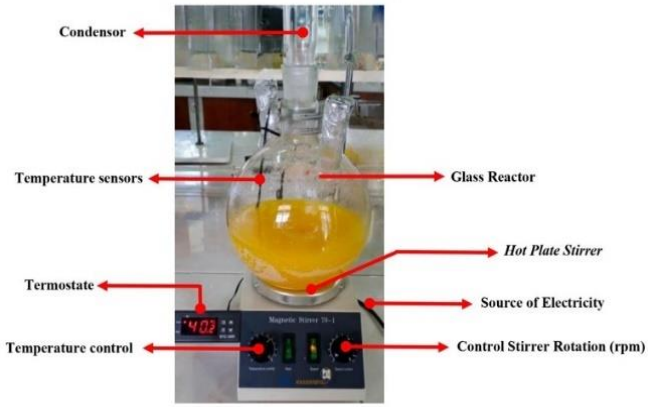


Figure 1. Synthesis of MCGs using CPKO and glycerol as substrates in a glass batch reactor

2.3 Experimental design

The optimization of the enzymatic transesterification to produce MCGs was conducted following Response Surface Methodology (RSM). RSM consists of a set of statistical techniques that facilitate the efficient optimization of processes that depend on several variables and provide a clear picture of the interactions between them [43]. The main advantage of RSM is the amount of data required for assessment, analysis, and optimization is less, thus requiring a smaller number of treatments. RSM responds to the shape of the surface curve for each independent variable efficiently examined with equal precision. One of the most widely recommended RSM data designs is the *Centre Composite Design* (CCD) [44-46]. This study involves three independent variables that follow the CCD matrix with 5 levels. The levels are indicated by coded values (-1.682; -1, 0, 1, +1.682) and actual values. The code level = 0, refers to the center point, while code level= (-1.682, -1), and (+1.682, +1) are low and high levels.

Table 1. Codified levels and actual value at CCD design

Variable (X)	Levels				
	-1.682	-1	0	+1	+1.682
Ratio of substrates (mole/mole)	1:2.32	1:3	1:4	1:5	1:5.68
CALB load (%wt)	0.12	0.15	0.20	0.25	0.28
Temperature (°C)	36.59	40	45	50	53.41

The CCD matrix was arranged with four replicates for each

level of the independent variables, resulting in 20 experiments. And then analyzed using RSM. This is aimed at obtaining an enzymatic transesterification condition that produces the optimal yield of MCGs by the interaction of 3 independent variables [44, 47]. The optimal reaction conditions of the variable response (Y) were obtained based on mathematical equations following a second-order polynomial [44, 47] as shown in Eq. (1):

$$Y=\beta_0 + \beta_1.X_1 + \beta_2.X_2 + \beta_3.X_3 + \beta_{11}.X_1 + \beta_{22}.X_2 + \beta_{33}.X_3 + \beta_{12}.X_1.X_2 + \beta_{13}.X_1.X_3 + \beta_{23}.X_2.X_3 \quad (1)$$

where, Y is the value of the variable response (yield of MCGs), β_0 is the intercept/constant, β_1 and β_2 are the linear coefficients, β_{11}, β_{22}, and β_{33} are the quadratic coefficients, β_{12}, β_{13}, and β_{23} are the variables interaction coefficient, where, ratio of substrate (X_1), CALB load (X_2), and reaction temperature (X_3) [48].

2.4 Anti-bacterial examination

The analysis consists of 4 steps: preparation of agar media and bacteria, inoculation of bacteria, and measurement of inhibition zone.

Preparation of agar media. 19 g of Mueller Hinton Agar (MHA) powder was added to 500 ml of distilled water and then heated until dissolved and homogenized. MHA media were sterilized in an autoclave at 121℃ for 15 min.

Preparation of *Escherichia coli* ATCC 25922 suspension. *E. coli* bacteria were taken ± 1 ose from Nutrient Agar (NA) solid media and placed in a test tube containing physiological NaCl solution. The suspension was homogenized by vortexing for 5 min. The turbidity of the *E. coli* bacterial suspension was adjusted to *Mc Farland* standard 0.5 ($\pm 1.5 \times 10^8$ CFU/ml). The bacterial suspension should be used as inoculum within 15 min.

Inoculation of *E. coli* Bacteria. Pour 20 ml of MHA medium into a petri dish (9 cm × 15 cm) and allow to solidify. Soak paper discs in MCGs solution for 30 min. *E. coli* bacteria are spread over the entire surface of the media using a sterile cotton swab. The soaked paper discs are then placed on a petri dish. Incubate at 37℃ for 5 × 24 h. As a positive control, we used gentamicin and aquadest as a negative control.

Measurement of inhibition zone. The anti-bacterial activity of MCGs was measured every 24 hours for 5 days. The inhibition zone was quantitatively measured as the area around the paper disc where there was no bacterial growth.

3. RESULTS AND DISCUSSION

3.1 Optimization of enzymatic transesterification

In this study, the enzymatic transesterification reaction between CPKO and glycerol occurred at an excess glycerol mole ratio to increase the chance of MCGs formation. Using of the same mole ratio between CPKO and glycerol resulted in a higher DGs composition than MGs. Transesterification is a reversible reaction, where the use of glycerol mole ratio can push the reaction rate towards the product so that excess glycerol molecules have the opportunity to bind fatty acids from CPKO [49]. The reaction mechanism is illustrated in Figure 2. The enzymatic reaction takes place in an organic solvent system. Hexane was used as the solvent because it was proven to increase the homogeneity of the substrate without interfering with the enzymatic activity of CALB. It uses the CALB lipase enzyme as a biocatalyst that works optimally on substrates containing MCFA [39]. CALB is immobilized in resin so that it is more stable in withstanding environmental temperature changes. Enzymes that have been immobilized on the resin can be used repeatedly after going through a purification process [50, 51]. In this study, fresh CALB was used for each experiment. Optimization of enzymatic transesterification involves three important independent variables, namely substrate mole ratio, enzyme CALB load, and reaction temperature. The substrate-mole ratio plays an important role in controlling the reaction speed of MCG formation. The CALB load plays a role in accelerating the reaction and catalyzing the transesterification reaction. Meanwhile, reaction temperature plays an important role in enhancing collisions between substrate molecules and controls the biological ability of CALB to survive changes in reaction temperature [52]. The interaction of these 3 independent variables is hypothesized to produce the optimal MCGs.

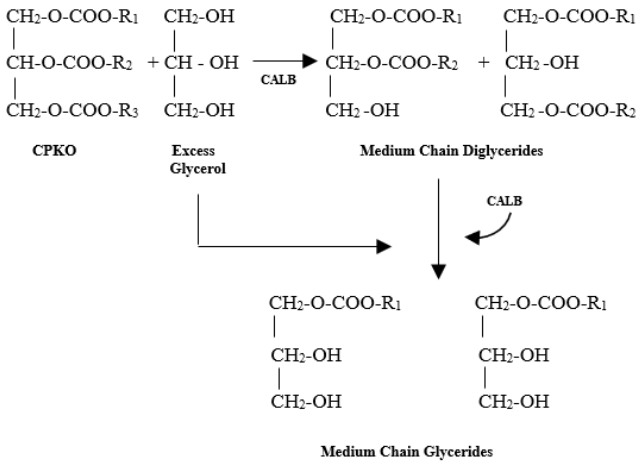


Figure 2. Schematic of the enzymatic transesterification between CPKO and excess glycerol using CALB

Other enzymatic reaction factors such as solvent ratio, stirrer rotation, and reaction time were used as fixed variables. The use of a solvent ratio of 1:3 (w/v) was able to reduce the interfacial tension between glycerol and CPKO and improve the homogeneity of the mixture. The stirring speed was used on a medium scale, 400 rpm. Stirring speed above 400 rpm can destroy the resin that coats the lipase enzyme, thus weakening the biocatalytic activity of CALB. Stirring speed below 400 rpm was not possible because the mixture of CPKO and glycerol has a high viscosity of ± 32 cSt. Although enzymatic reactions always have long reaction times [53], in this study, the reaction time was set at 480 minutes in consideration that other variables also influence the yield of MCGs. The utilization of the 3 independent variables in the optimization of enzymatic transesterification had more influence on increasing the yield of MCGs than increasing reaction time. A long reaction time causes a decrease in MCGs yield because CALB naturally enters a stationary phase characterized by a decrease in biocatalytic activity.

The results of optimization research on 20 trials with different treatments showed that an optimal MCGs yield of 82.85% was obtained in trial 1. All MCGs produced from the optimization of enzymatic transesterification reaction contained a higher percentage of MGs than DGs. From the 20 trials conducted, the optimal reaction conditions were obtained

at a substrate ratio of 1:3 (mole/mole), a CALB load of 0.25% wt, and a reaction temperature of 40°C. The results of the optimization study are shown in Table 2. Furthermore, the RSM analysis provided a prediction of reaction conditions that can produce an MCGs yield of >82.85% through the interaction of the 3 independent variables. The predicted reaction conditions are presented in the form of a quadratic regression equation model (Eq. (2)). The results of the statistical analysis of 3 independent variables are shown in Table 3 and Table 4.

Table 2. Experimental condition of CCD and corresponding response (experimental results)

Trial	Level of Variables (X)			Response
	X_1	X_2	X_3	Yield of MCGs
1	- 1	- 1	- 1	82.85
2	+1	- 1	- 1	73.37
3	- 1	+1	- 1	68.93
4	+1	+1	- 1	77.36
5	- 1	- 1	+1	77.54
6	+1	- 1	+1	75.88
7	- 1	+1	+1	67.09
8	+1	+1	+1	73.32
9	-1.682	0	0	80.17
10	+1.682	0	0	81.76
11	0	-1.682	0	69.68
12	0	+1.682	0	63.28
13	0	0	-1.682	70.96
14	0	0	+1.682	60.41
15	0	0	0	69.50
16	0	0	0	71.65
17	0	0	0	71.22
18	0	0	0	69.69
19	0	0	0	70.50
20	0	0	0	70.88

Table 3. Statistical analysis for independent variables

Parameters	Statistical Analysis	
	Coefficient	P-Value
Constant	70.46	0.00
Ratio of substrate (X_1)	0.45	0.55
CALB load (X_2)	-2.47	0.01
Temperature (X_3)	-1.93	0.02
X_1*X_1	4.41	0.00
X_2*X_2	-0.72	0.33
X_3*X_3	-0.99	0.19
X_1*X_2	3.22	0.01
X_1*X_3	0.70	0.48
X_2*X_3	-0.39	0.69

The regression equation was obtained based on ANOVA and its accuracy was measured by the R^2 determinant. Based on the analysis results, R^2 = 88.48% and R (adj) = 78.11% were obtained for the regression equation. This shows that 88.48% of the response is influenced by the independent variables involved and the optimization. The R^2 value reflects the accuracy of the resulting equation model. There are two suitable regression equation models were obtained for MCGs synthesis using independent variables X_1, X_2, X_3. The F-value = 6.37 for linear regression with p-value = 0.011 (α = 0.05). For quadratic regression, F-value = 15.12 with P-value = 0.0 (α = 0.05). Among the two models, the quadratic regression equation has the lowest P-value.

The quadratic regression equation is more accurate in describing the effect of the independent variables on the response variable than linear regression. The quadratic regression equation is shown below:

$$Y=70.46+0.45.X_1-2.47.X_2-1.93.X_3+4.41.X_{12}-0.72.X_{22}-0.99.X_{32}+3.22.X_1.X_2+0.70.X_1.X_3-0.39.X_2.X_3 \quad (2)$$

The quadratic regression equation has been examined through a model normality test based on lack of fit (LOF), using this hypothesis:

H_o: There is no lack of fit

H_1: There is a lack of fit

The ANOVA result in Table 4 shows that LOF < 0.05 (P-value = 0.003) indicates that the regression model has represented the entire data analysis.

Table 4. Result of analysis of variance (ANOVA)

Source	DF	Sum of Square	Mean Square	F-Value	P-Value
Model	9	550.29	61.14	8.53	0.00
Linear	3	136.99	45.67	6.37	0.01
X_1	1	2.79	2.79	0.39	0.55
X_2	1	83.13	83.13	11.60	0.01
X_3	1	51.06	51.06	7.13	0.02
Square	3	325.01	108.34	15.12	0.00
X_1*X_1	1	279.75	279.75	39.04	0.00
X_2*X_2	1	7.37	7.37	1.03	0.33
X_3*X_3	1	14.29	14.29	1.99	0.19
2-Way Interaction	3	88.28	29.43	4.11	0.04
$X_1.X_2$	1	83.17	83.17	11.61	0.00
$X_1.X_3$	1	3.92	3.92	0.55	0.48
$X_2.X_3$	1	1.19	1.19	0.17	0.69
Lack of Fit	5	68,066	13,613	18,92	0.003
Error	10	71.66	7.17		
Pure Error	5	3.59	0.72		
Total	19	621.95			

3.2 Interaction between ratio of substrates (X_1) and CALB load (X_2) to increase MCGs yield

Observations of the ratio of substrates (X_1) and CALB load (X_2) were carried out at a reaction temperature (X_3) of 45°C (code value = 0). The RSM shows that the surface response expresses a deep curve towards level 0 for variable X_1 which indicates a decrease in MCGs yield as the ratio of substrates increases. Meanwhile, the effect of increasing X_2 compared to X_1 on MCGs yield shows a wider and higher curve. This shows that increasing the concentration of CALB has a significant effect on increasing MCGs yield. The response surface plot (Figure 3(a)) shows that the optimal MCGs yield is not at the center point level, but between the low (-1.682) and high (+1.682) levels. The influence of X_1 is between levels (-1.682) to (-0.5) or levels (+1) to (+1.682). Meanwhile, X_2 influences the increase in MCGs yield at a broader level, namely (-1.682) to (+1.682). The interaction between X_1 and X_2 at this level produces a higher MCGs yield. The level range of X_2 is wider than X_1, so the influence provided by X_2 is greater than X_1. This is reinforced by the regression analysis results showing that the p-value of X_2 is more significant than X_1 (P-value = 0.01). The interaction between X_1 and X_2 shows a significant influence on the yield of MCGs.

Contour plots (Figure 3(b)) provide a more detailed approach to the actual values of the independent variables. To observe the influence of X_1 and X_2, the contour area plotted X_3

at code value = 0. Contour plot displays the areas where the model provides optimal prediction values. The red contour area is the area where the level of the independent variable is predicted to provide an MCGs yield of 65-75%. While the green contour areas give prediction MCGs yield of >75%.

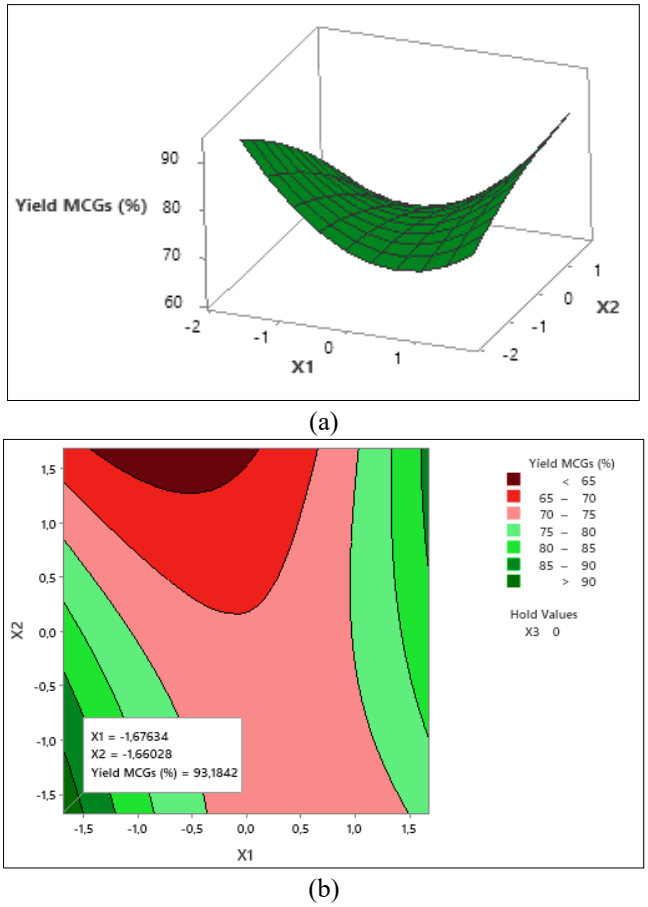


Figure 3. Interaction of ratio of substrate (X_1) and CALB Load (X_2) at temperature = 45°C (a) Surface response plot, (b) Contour plot

Contour analysis has identified 2 areas (green area) for MCGs yield of ≥ 75-90%. In the codified level, the first area is at X_1 = > -1.5 to 0.5, X_2 = > -1.5 to <1.0, and the second area is at X_1 = >1.5, X_2 = >-1.5 to >1.5. The variables process (prediction) based on RSM is X =-1.67634 (actual value = 1:2.32 mole), X_2 = -1.66028 (actual value = 0.12% wt), X_3 = 0 (actual value = 45°C) with a yield of 93.18%. Surface analysis shows that there is a limited interaction between X_1 and X_2. Limitations on the interaction of these 2 independent variables occur due to substrate limitations.

The use of X_1 and X_2 at code level = > 0 resulted in optimal MCGs yield. An increase in CALB load has to be followed by an increase in the ratio of substrates to avoid substrate limitation. Substrate limitation causes the enzyme-substrate (ES) complex not to be formed, so the enzyme does not perform biocatalytic activity. This is consistent with other studies that have found that using a high enzyme load with a low substrate concentration can cause enzyme aggregation and reduce the yield of MCGs [38, 54].

3.3 Effect of ratio of substrate (X_1) and temperature reaction (X_3) to MCGs yield

The response surface plot (Figure 4(a)) illustrates the increase in MCGs yield as the curve rises towards level X_1 > 0. This is confirmed by statistical analysis which shows a positive regression coefficient value of 0.45 even though it is not significant (P-value = 0.55). Meanwhile, the surface response expression of reaction temperature shows a more stable curve that tends to flatten as the reaction temperature increases (X_3). This shows that the influence of reaction temperature on the reaction is on a wide level. So the reaction temperature as a single independent variable does not have a significant influence on the MCGs yield. The interaction X_1 and X_3 does not have a significant effect on increasing MCGs yield because the X_3 level is wider than X_1. This confirms that the reaction can occur at moderate temperatures, at the codified level < 0, or (actual value = < 45°C).

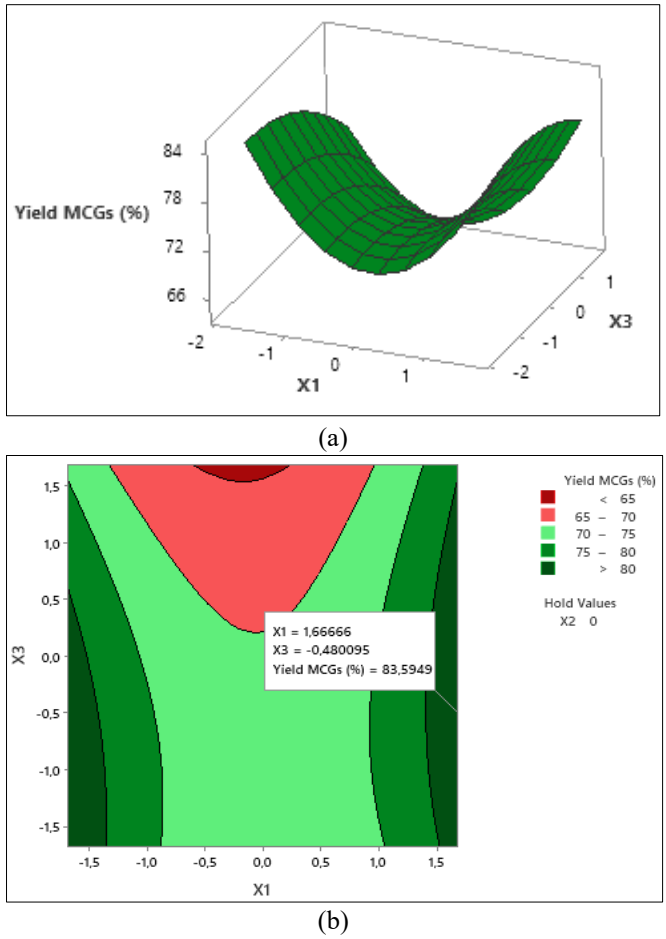


Figure 4. Interaction of ratio of substrate (X_1) and reaction temperature (X_3) at CALB load 0.20% wt (a) Surface response plot, (b) Contour plot

Based on contour area plot analysis, it is known that the interaction of X_1 and X_3 at code level = 0 (center point) is predicted to produce an MCGs yield of only 65-70% (red area). The interaction of the 2 independent variables at level 0 does not have a significant effect on increasing MCGs yield. However, the area contour plot also provides predictions of enzymatic transesterification reaction conditions with a chance of MCGs yield > 70%. The first contour area (green area) is plotted at the codified level < 0, where the X_1 level is in the range (-1.68090) or (actual value = 1:2.32 mol) to (-0.620375) or (actual value = 1:3.38). The second area is at the codified level X_1 > 0, where the X_1 level is in the range (0.620375) or (actual value = 1: 4.62) to 1.68090 (actual value = 1: 5.68). Meanwhile, X_3 plotted at -1.68090 (accrual value =

36.6°C) to 1.68090 (actual value = 48.4°C). The optimal yield (prediction) for this condition is X_1= 1.66666 (actual value = 1:5.67 mole/mole), X_3 = -0.480095 (actual value = 37.6°C), and X_2 = 0.2% wt with a yield of 83.59%.

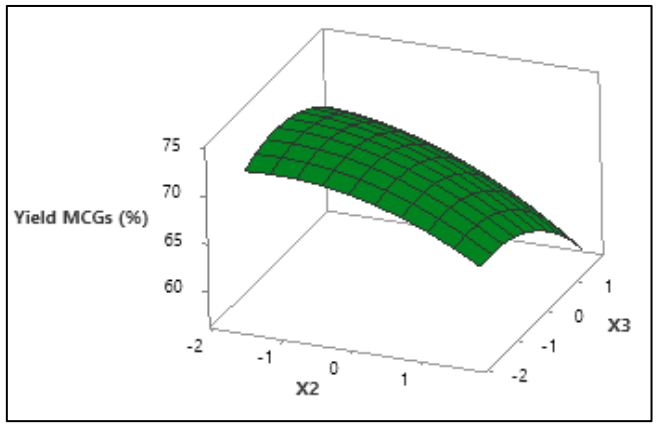
According to RSM analysis, it is known that the interaction between X_1 and X_3 in X_2 at 0.2% wt has no significant effect on the reaction. It shows that increasing X_1 and X_3 cannot increase the reaction speed towards the product at a fixed enzyme load. The reaction speed would decrease because the ratio of substrates increases but is not accompanied by an increase in the CALB load, even though the reaction temperature is increased. Even using a reaction temperature > 45°C still reduces the yield of MCGs [52, 55, 56]. Another research [57] showed that high ratio of substrate led to decrease in mass transfer due to an increase in substrate viscosity, resulting in a lower product yield.

3.4 Effect of *Candida antarctica* (CALB) load (X_2) and reaction temperature (X_3)

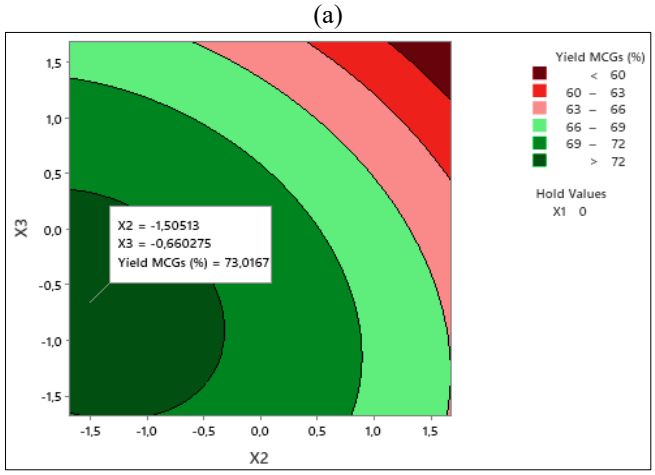
The effect of CALB load (X_2) and reaction temperature (X_3) was observed at a ratio of substrate 1:4 (mole/mole) or (code value = 0). The independent variable plot shows a visualization of the descending surface. The increase in CALB load has the opposite effect on the product. MCGs yield decreases as X_2 increases. The response surface (Figure 5(a)) shows the influence of X_2 and X_3 on the product. MCGs yield decreased as substrate concentration increased. The interaction between CALB load (X_2) and reaction temperature (X_3) has no significant effect with a regression coefficient of -0.385. The negative and insignificant regression coefficient value confirms that increasing MCGs yield is obtained at variable levels X_2 and X_3 at < 0. This is reinforced by the results of statistical analysis from X_2 which shows a regression negative coefficient of -2.47. This illustrates that the increase in X_2 level is not linear with the increase in MCGs yield. The reaction temperature (X_3) shows a curve with the peak of the curve at a temperature of 45°C (coded value = 0). Using a reaction temperature greater or less than 45°C showed a decrease in MCGs yield, although it was not significant. This condition is reinforced by statistical analysis which shows a regression negative coefficient for the enzymatic reaction -1.934, indicating that the increase in reaction temperature is not linear with the increase in MCGs yield.

The area contour (Figure 5(b)) shows the levels of independent variables that provide optimal MCGs yields (green area). The first contour area shows a predicted MCGs yield of 66-72%. In this condition, X_2 is used at codified level < -1 (actual value = 0.10% w/w) to 1.68090 (actual value = 0.28% wt). For X_3, it falls within a wide interval of -1.5 - 0.4807 (actual value = 37.5 - 47.4°C). The second contour area for MCGs yield ≥ 73% is shown with X_2 at the level ≥ -1.50513 (actual value = ≥ 0.05% wt) to -1 (actual value = 0.10% wt), and X_3 at codified value = ≥ -1.68090 (actual value = ≥36.6%) to ≤ 0.5 (actual value = ≤47.5°C). The optimal yield (prediction) by the counter area, plotted at X_2 = -1.50513 (actual value = 1:2.49 mole/mole), and X_3 = -0.660275 (actual value = 41.7°C) with a yield of MCGs at 73.02%.

The expression of the surface response and area contour depict the same effect, namely increasing the levels of X_2 and X_3 results in a decrease in MCGs yield. Increasing the CALB load without being accompanied by an increase in substrate concentration will not increase product yield. This is because the available substrate has been completely broken down by the enzyme into the product so increasing the lipase concentration has no impact on the product. In addition, increasing the reaction temperature can increase lipase activity to break down the substrate. However, because the available substrate is limited, the reaction speed decreases [58].



(a)



(b)

Figure 5. Interaction of *Candida antarctica* Load (X_2) and reaction temperature (X_3) at substrate concentration 1:4 (mole/mole). (a) Surface response plot, (b) Contour plot

Based on the area contour, it is predicted that controlling the interaction between X_1 and X_2 can produce MCGs yield of ≥ 93%. Meanwhile, controlling for the interaction between X_1 and X_3 and X_2 and X_3 only resulted in an MCGs yield of ±73-83%. As confirmed by ANOVA, the regression coefficient for X_1 is 0.45, indicating a linear effect between X_1 and the increase in MCGs yield. Meanwhile, the regression coefficient for CALB load (X_2) is -2.47 and the reaction temperature (X_3) is -1.93. This indicates that an increase in X_2 and X_3 leads to a decrease in MCGs yield. The interaction between X_1 and X_2 and X_2 and X_3 resulted in reaction coefficients of 3.22 and 0.70 respectively, indicating that an increase in X_1 followed by an increase in X_2 is linearly related to an increase in MCGs yield. In contrast, the X_1 and X_3 interaction was -0.39, indicating that an increase in reaction temperature and substrate ratio resulted in a decrease in MCGs yield. This proves that CALB works optimally at moderate temperatures, following previous studies [59, 60] which obtained the highest reaction conversion at 40-50°C. The utilizing of reaction temperatures >50°C causes the denaturation of CALB, and reduces catalytic activity [57, 61]. Here are some previous reports on mono-di-glycerides optimization research using different substrate sources (Table 5).

Table 5. Comparison of MGs and DGs yield for enzymatic transesterification optimization using lipase enzyme as biocatalyst

Substrate	TGs:Gly (mole)	Lipase (%wt)	Temp (°C)/ Time (h)	Solvent	Main Product		Lipase Source	Reference
					MGs (%)	DGs (%)		
Crude palm kernel oil (CPKO)	1:3	0.15	40/8	Hexane	57.61	25.24	*Candida antarctica*	Present Paper
Lauric acid	1:4	4.0	60/3	Solvent-free	50.00	34.60	*Rhizomucor meihei*®	[24]
Oleic acid	1:1	5.8	75/3	Ethanol	38.71	39.45	*Novozyme 435*®	[52]
Soybean oil	1:6	15	70/12	Solvent-free	28.30	-	*Novozyme 435*®	[38]
Olive oil	1:2.8	0.25	40/5	Solvent-free	38.71	39.45	*Candida rugosa*	[62]
Linseed oil and Oleic acid	1:3.36	4.0	57.7/48	Hexane	24.66	48.57	*Novozyme 435*®	[63]
Glycerol trioleate	1:3	0.9	35/24	Aseton, Isooktan	23.98	16.82	*Rhizomucor meihei*®	[64]
Palm fatty acid distillate (PFAD)	1:1	3.0	50/24	Solvent-free	23.90	39.80	*Rhizomucor meihei*®	[65]
Babassu oil	1:15	10	55/6	Solvent-free	25.00	70.0	*Burkholderia cepacia*	[66]
Sunflower oil	1:6	10	50/60	Tert-butanol, Tert-pentanol	37.80	8.64	*Candida antarctica*	[67]
Triolein	1:1	10	60/2	H_2O	19.20	21.40	*Rhizomucor meihei*®	[68]
Refined olive residue oil	1:2	18	30/24	Hexane	32.00	18.00	*Candida rugosa*	[69]

3.5 Profile of medium chain-glycerides

3.5.1 Composition of optimal medium chain-glycerides

Based on GC analysis of the optimal MCGs at trial 1 (Figure 6), the first peaks detected were mono-caprate and mono-caprylate. The peak with the highest intensity was mono-laurate which was detected at a retention time (rt) of 10-12 min, followed by mono-myristate at rt of 13-14 min [70, 71]. Hydrogen bonds from MCFAs are broken and bind to glycerol molecules that CALB has hydrolyzed and form MCGs. Optimal MCGs consisted of mono-caprate at 2.14%, mono-caprylate was 1.52%, mono-laurate was 47.08%, and mono-myristate was 3.28%. The total yield of perfectly reformed MCGs was 54.02%.

CPKO contains 77.41% of MCFA. Enzymatic transesterification which is catalyzed by CALB successfully converted 69.78% of MCFA into MCGs. CPKO also contains 22.58% long-chain fatty acid (LCFA). LCFAs were converted into long-chain glycerides (LCGs), consisting of mono-palmitate 2.84%, mono-stearate 0.75%, and the rest were detected as DGs. DGs peaks started to be detected at 21-25 min with intensity below the mono-laurate peak, which illustrates that the composition of MGs is higher than DGs. DGs consist of 2 alkyl groups from fatty acids and one hydroxyl group bonded to 1 molecule of glycerol. The use of excess glycerol is needed to form the MGs bond. The total yield of MGs was 57.61% (54.02% MCGs, and 3.59% LCGs), and the total yield of DGs was 25.24%. MCGs consist of MGs and DG which contain a mixture of fatty acids consisting of CPKO. MCGs has characteristics close to their origin fatty acids, so can be widely applied in various food products. The optimal MCGs containing residual glycerol were detected at 0.80-0.87 min with very low intensity, indicating that the excess glycerol at a substrate ratio of 1:3 (mole/mole) had properly reacted with CPKO. The chromatogram also detected the presence of TGs detected at rt 25.6 min [72]. TGs are unreacted CPKO data derived from single and double-chain LCFA (≥18 carbon chains) that cannot be reformed, because CALB is not selective on LCFA. The MCGs chromatograms for trials 2 to 20 showed similar MGs and DGs profiles to trial 1, with peak intensities varying according to the amount of converted MGs and DGs. The optimized MCGs product met the Food Agricultural Organization (FAO) standard which required an MGs content of ≥30% for commercial MCGs [73].

The analysis results prove that MCGs consist of MGs and DGs, which mixture of fatty acids from CPKO. The characteristics of MCGs are close to their origin of fatty acids [3]. Different fatty acids that are used as a substrate, would produce an emulsifier with a different hydrophile lipophile balance (HLB) [31, 74]. The application area of an emulsifier is determined based on the HLB value [5]. The optimal MCGs have an HLB value of 3.55 and are classified as water-in-oil. Emulsifiers have an HLB range of 1-20 [75]. MCG has a low HLB so it can disperse water into emulsion systems that have a high concentration of oil or fat [76]. These emulsion systems are found in many food products produced with the addition of water and oil or fat as the main ingredients, such as bread, cakes, pasta, frozen desserts, icing, toppings, peanut butter, margarine, shortening, dairy products, and coffee whitener [77, 78].

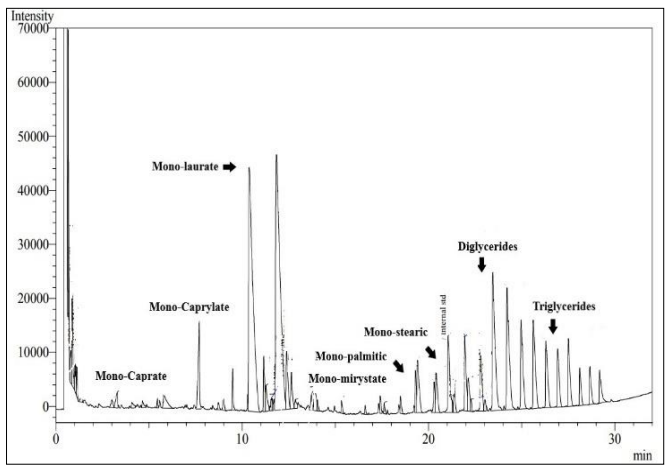


Figure 6. GC chromatogram of the optimal MCGs

3.5.2 Medium chain-glycerides functional groups

MCGs are esters of triglycerides that are easily soluble in oil or fat because it has an alkyl group from the hydrocarbon chain of fatty acids that make up triglycerides. But MCGs also have solubility in H_2O because they contain 2 hydroxyl groups in their molecule. MCGs have 2 specific main functional groups, differentiating them from other triglyceride derivative compounds. MCGs have a hydroxyl group (-OH) at an absorption wave number 3,273.7 cm^{-1}, while CPKO does not have a hydroxyl group [79]. The absorption intensity of the hydroxyl group in MCGs was detected as strong with a sharp curve and not widening.

Table 6. Wave number of optimal MCGs and CPKO

Functional Groups	Wave Number (cm^{-1})	
	MCGs	CPKO
-OH	3,273.7	-
-CH	2,880.1	2,853.2
$-CH_2$	2,931.7	2,921.6
-C=O	1,742.9	1,742.5
-CO	1,211.9-1,127.2	1,480.8
-C-O-C	1,414.9-1,031.7	1,154.7-1,1093.4

The curvature of the -OH group which does not widen indicates the existence of hydrogen bonds between MCGs molecules which are arranged in mixed fatty acids. The width of the -OH absorption band can differ depending on the type of fatty acid bound to the -OH group. The second main functional group is the carbonyl group, namely C=O which indicates the presence of an ester bond. CPKO has a C=O wavenumber of 1,742.52 cm^{-1} with a short and wide absorption band. The C=O group indicates the ester bond comes from mixed fatty acids. The C=O functional group in MCGs was detected at a wave number of 1,742.9 cm^{-1}, with a wide absorption band which proves that MCGs consist of more than one type of fatty acid. Another supporting functional group is -CH which is a functional group that is always present in organic compounds [80]. The -CH group for CPKO was detected in the absorption area of 2,853.22 cm^{-1}, while MCGS was detected at 2,880.1 cm^{-1}. The -CH absorption band on CPKO looks sharper than MCGs. CPKO has many -CH bonds compared to MCGs, which originate from the long fatty acid hydrocarbon chain. The wave numbers of the functional groups that appear in MCGs and CPKO are presented in Table 6.

In addition to the -OH, -CH, $-CH_2$ and C=O groups, four functional groups indicate that MCGs are a derivative of CPKO and glycerol. CPKO has a $-CH_2$ group at 2,931.7 cm^{-1} (strong), because triglycerides bind three fatty acid molecules with different carbon chain lengths. MCGs also have a -CH group at 2,921.6 cm^{-1} according to the type of fatty acid being bound. CPKO has one functional group of $-CH_2$ (stretching) band spectrum, whereas in MCGs there are two $-CH_2$ curved bands due to the influence of the -OH group. In CPKO, the -CO functional group was detected at 1,480 cm^{-1} with a sharp intensity (stretching). However, in MCGs the -CO group has a wider and stronger stretching vibration. The -C-O-C functional group is a natural characteristic of all triglycerides and their derivatives.

The detection of the functional group of -C-O-C at 1,414.9 cm^{-1}-1,031.7 cm^{-1} indicates that the MCGs molecule is composed of more than one type of MCFA. Based on the comparison of the spectrum between MCGs and CPKO, there has been a significant change in molecular structure from triglycerides and glycerol to MGs and DGs through enzymatic transesterification reaction by CALB catalysis. Similar analytical results were obtained in study [20], where MGs and DGs were synthesized from palm oil at a substrate ratio of 1:6 (mole/mole) using *Candida sp.* at 40°C for 24 h. The wave number of MCGs and substrates can be seen in Figure 7.

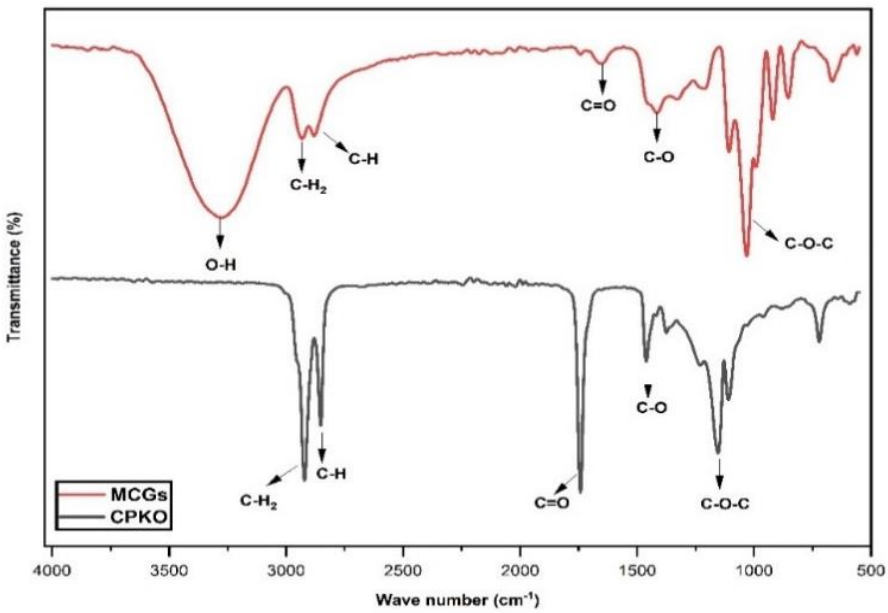


Figure 7. Comparison FTIR spectrum of MCGs and CPKO

3.6 Anti-bacterial activity of mono-diglycerides against *Escherichia coli*

MCGs synthesized from CPKO contain high MCFA which is known to have anti-bacterial properties. An examination of the anti-bacterial properties was conducted to identify whether the MCGs still have the original properties that belong to MCFAs after going through enzymatic transesterification. The selection of bacteria is based on pathogenic properties and the medium of infection. Gram-negative bacteria are more

pathogenic than gram-positive, and cause many diseases that can be infected to humans through food products [81, 82].

The optimal MCGs were examined on the gram-positive pathogenic bacteria *Escherichia coli* (*E. coli*). Gram-negative bacteria (such as *E. coli*) have a double membrane system, where the plasma membrane is enveloped by a permeable outer membrane. These bacteria have a more complex structure, consisting of three-layered cell walls, namely the outer layer of lipoproteins, the middle layer of lipopolysaccharides, and the inner layer of peptidoglycan which makes it difficult to penetrate by anti-bacterial compounds. The following is an anti-bacterial activity analysis for 5 days of observation, shown ini Table 7.

Table 7. Anti-bacteria activity analysis using different MCGs concentrations

Concentration of MCGs	Inhibition Zone (mm)-Days					
	1	2	3	4	5	Av
Control (-)	0	0	0	0	0	0
Control (+)	25.1	26.3	25.2	25.1	24.9	25.3
1 mg/mL	7.1	7.2	10.1	11.7	11.7	9.6
2 mg/mL	10.3	9.9	11.8	11.7	11.4	11.0
3 mg/mL	10.1	10.7	11.7	11.8	11.3	11.1
4 mg/mL	8.5	8.8	10.8	13.6	14.1	11.2
5 mg/mL	8.3	8.4	9.3	13.4	14.0	10.7

Control (-) = aquadest, Control (+) = gentamycin, Av = average.

Based on the analysis results, it is proved that MCGs can inhibit the activity of *E. coli*. The ability of MCGs can inhibit bacterial activity increased during the incubation period. Application of MCGs at a concentration of 2-4 mg/mL showed the highest inhibition zone compared to other concentrations. These results are in agreement with previous research, which conducted anti-bacterial analyses on five types of MCGs. MCGs were able to inhibit the activity of gram-negative bacteria at a concentration of 2.5 mg/mL for mono-myristate [28]. The inhibition zone of MCGs for 5 days incubation is shown in Figure 8.

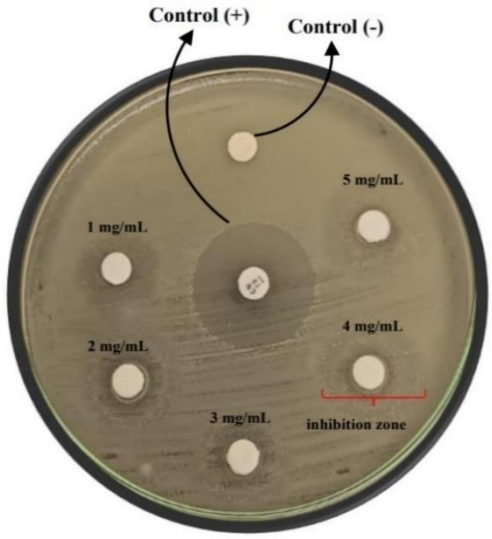


Figure 8. The appearance of the inhibition zone in *E. coli* (MCGs concentration of 4 mg/mL, for 5 days incubation)

The anti-bacterial activity in MCGs comes from mono-caprate, mono-caprylate, mono-laurate, and mono-myristate which is known to inhibit the growth of microorganisms [3]. This condition proves that enzymatic transesterification, which takes place at a moderate temperature (40-45°C), can maintain the natural anti-microorganism characteristics of MCFA in CPKO. Determination of the anti-bacterial activity of the MCGs was measured through the radius of the zone of inhibition. The zone of inhibition is the area where there is no bacterial growth. Based on the radius of the inhibition zone, it is known that MCGs have bacteriostatic properties because can inhibit the growth of *E. coli*, so that the bacteria remain stationary or do not multiply.

The anti-bacterial properties of MCGs enhance their primary function as an emulsifier. MCGs have the potential ability to prevent food spoilage caused by microorganisms, thereby increasing shelf life. In addition, the application of MCGs in food products ensures food security for consumers [83, 84].

4. CONCLUSIONS

The optimal MCGs product was obtained at a substrate concentration of 1:3 mole (CPKO/glycerol), CALB load at 0.15% wt, and a temperature of 40°C. MCGs consist of MGs and DGs yields are 57.61% and 25.24%, respectively. Based on ANOVA result, the enzymatic transesterification reaction is influenced by the interaction of substrate concentration (X_1) with CALB load (X_2) with a regression coefficient of 3.22 with P-value=0.01 (sig. ≤ 5%). Area contour based on RSM analysis predicts that enzymatic transesterification carried out at X_1=-1.67634 (actual value=1:2.32 mol/mol), X_2=-1.66028 (actual value=0.12 wt%), X_3 = 0 (actual value = 45°C) gives a yield of 93.18%. The optimal MCGs have 6 functional groups -OH, -CH, $-CH_2$, -C=O, -CO, and -C-O-C. There are significant differences in the functional groups between MCGs and CPKO (substrate) which is used as a comparison. MCGs have an -OH functional group with a (strong) bend in the absorption band at 3,273.7 cm^{-1}. The -OH functional group indicates that an ester bond has been formed between glycerol and fatty acids from CPKO to form MCGs. The optimal MCGs are known to have anti-bacterial properties. Identification of the anti-bacterial activity shows that the application of MCGs is bacteriostatic, which can inhibit the growth of *E. coli* gram-negative bacteria. The highest zone of inhibition was 11.2 mm, obtained at a concentration of 4 mg/mL for an incubation period of 5 days. Based on the studies conducted, MCGs are classified as water-in-oil emulsifiers with an HLB of 3.55 and have the additional characteristic of being anti-bacterial. This research opens up opportunities for the synthesis of emulsifiers from other triglyceride sources that have hidden potential properties as anti-bacterial. Further study can focus on the application of MCGs in processed food to determine the performance of MCGs that are synthesized from CPKO in preventing food damage by microorganism activity.

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