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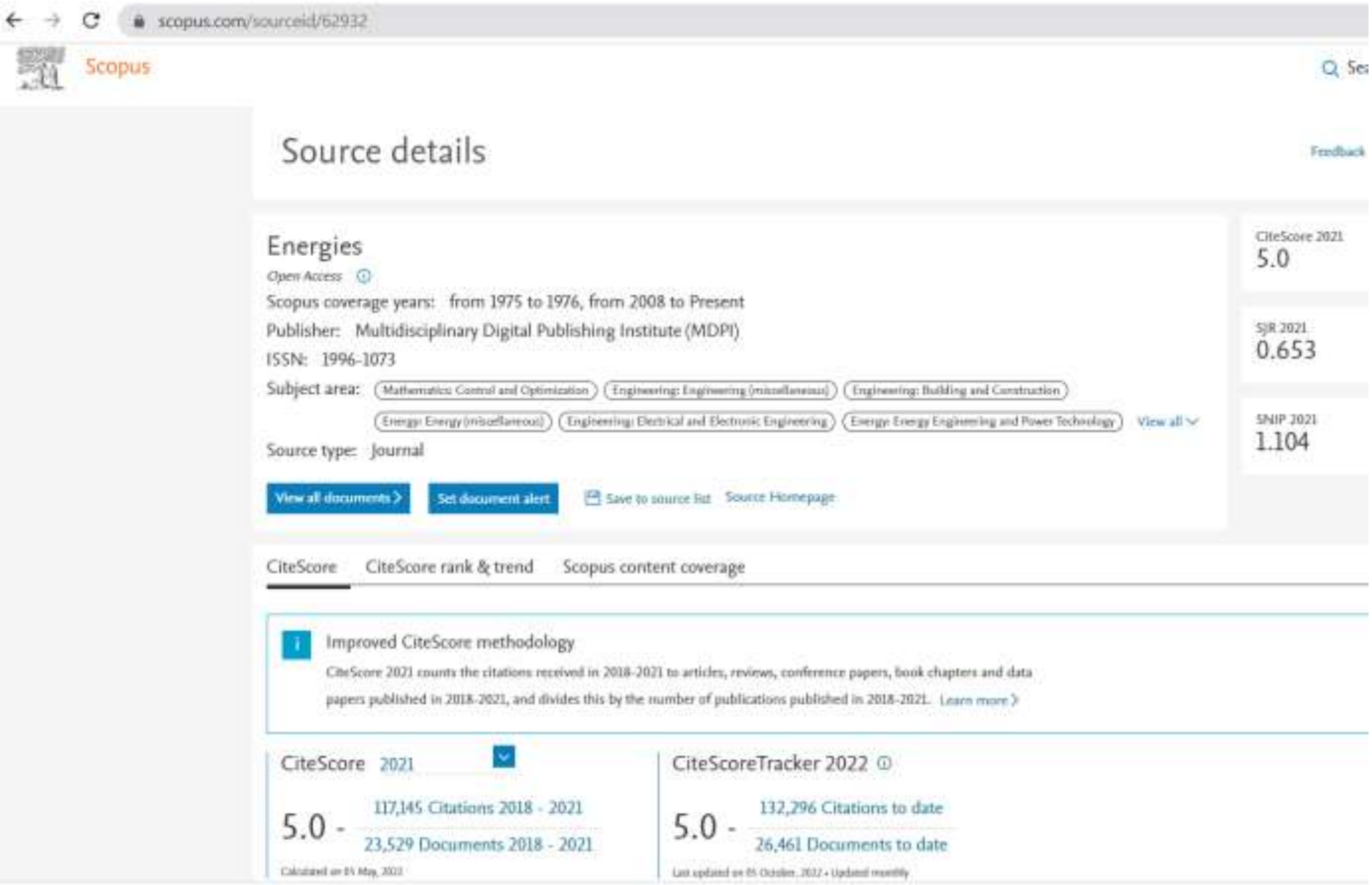

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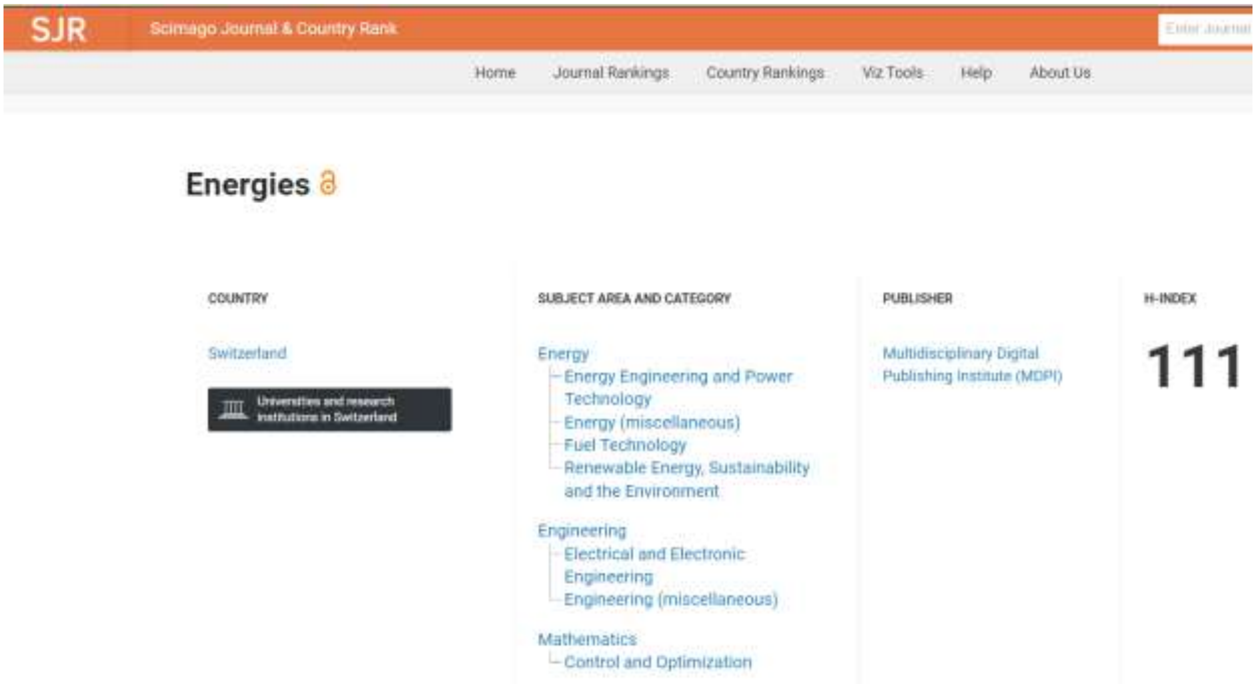

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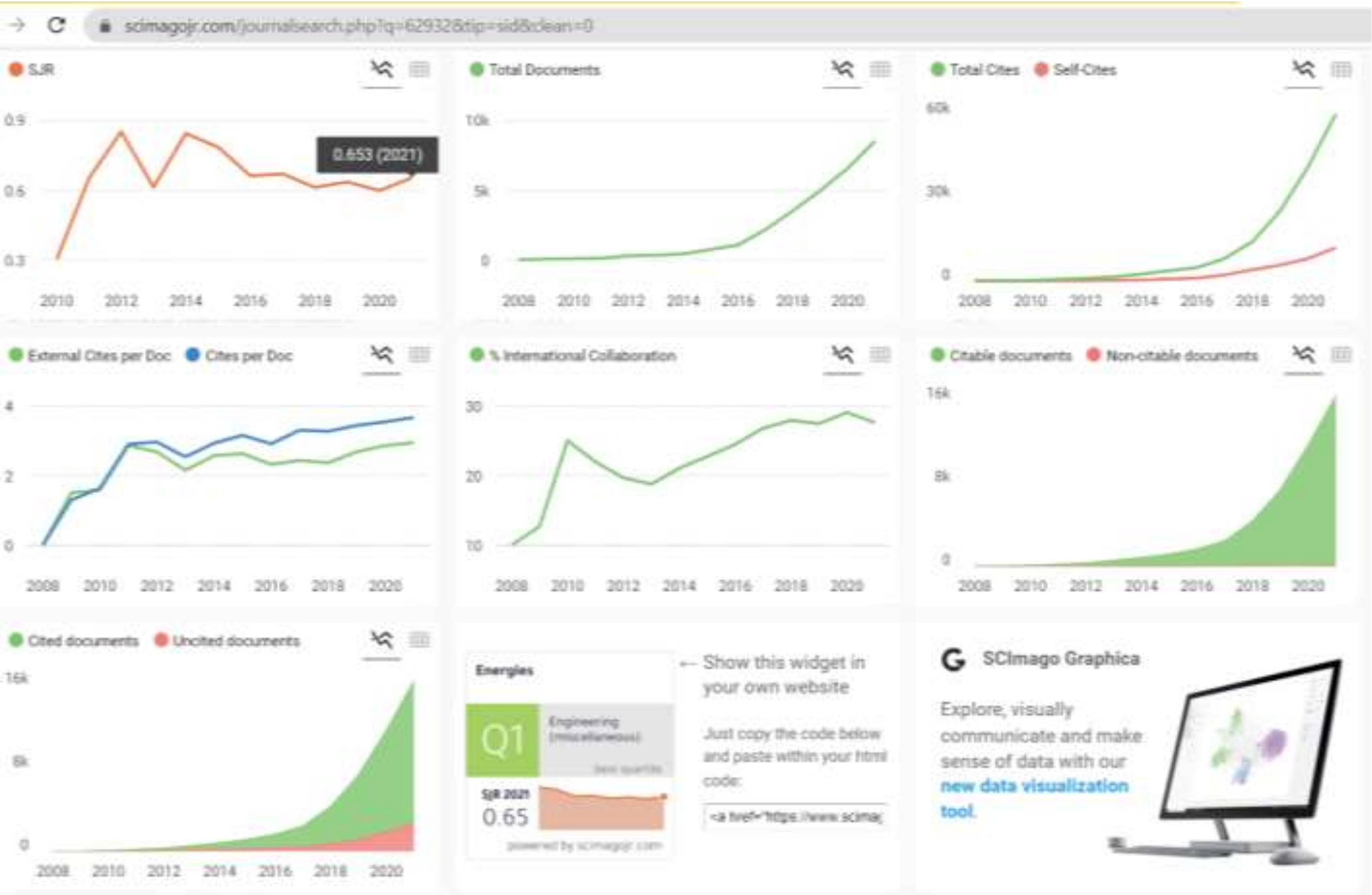

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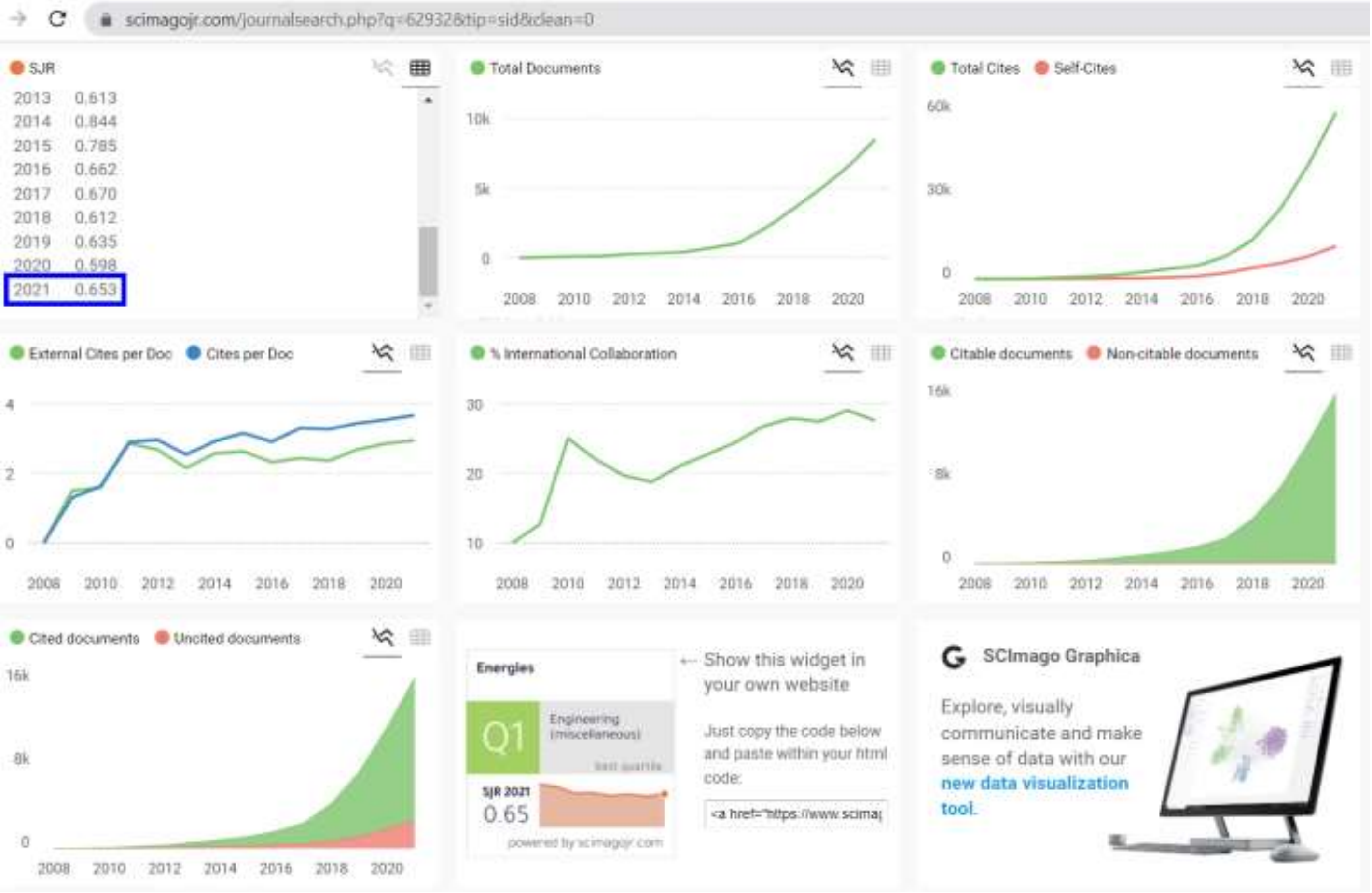

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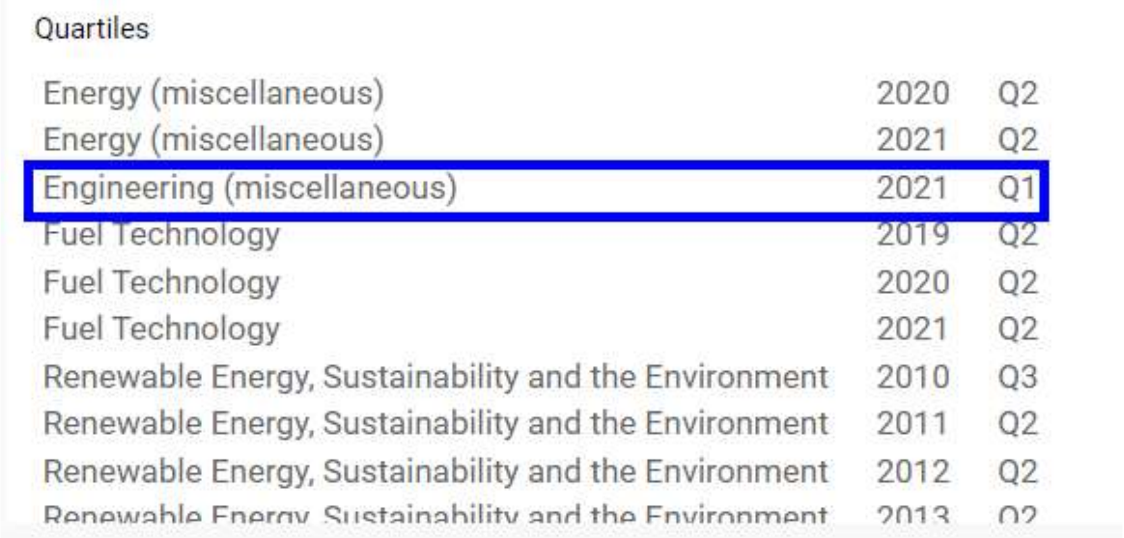

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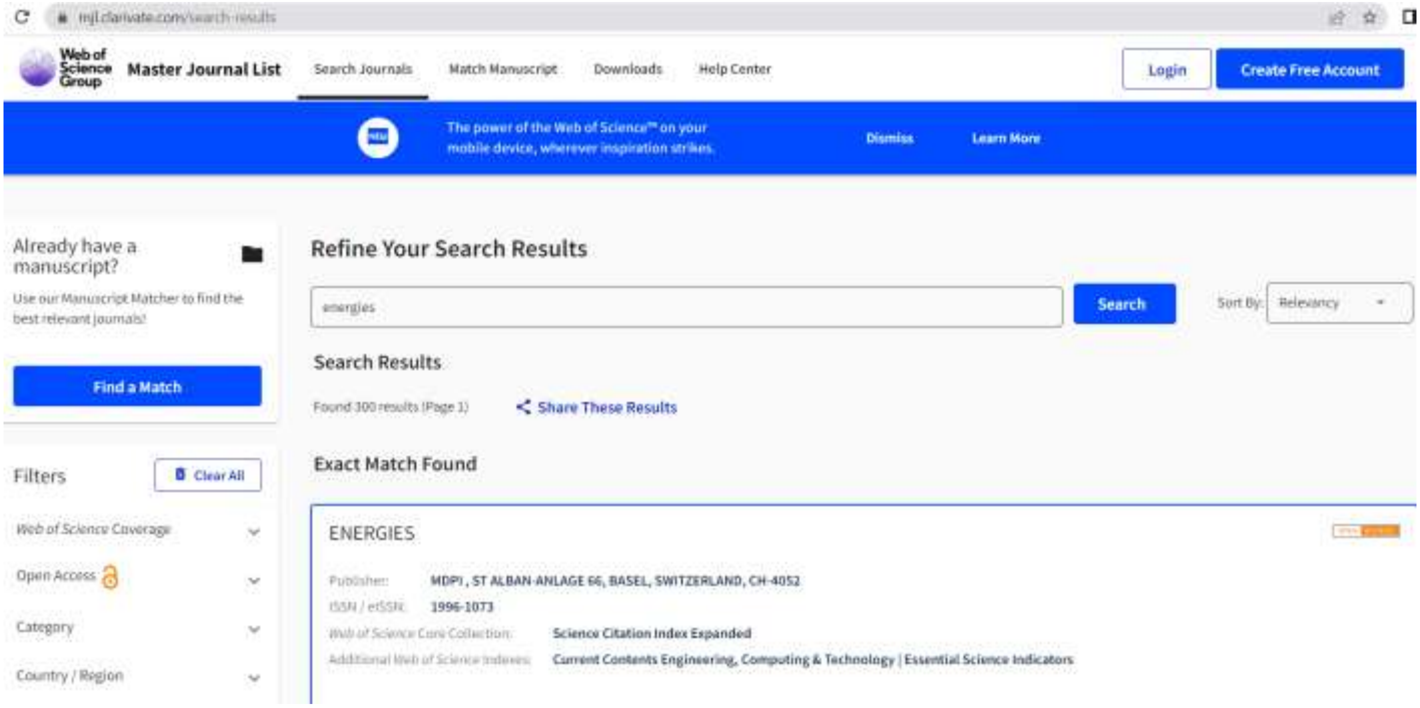

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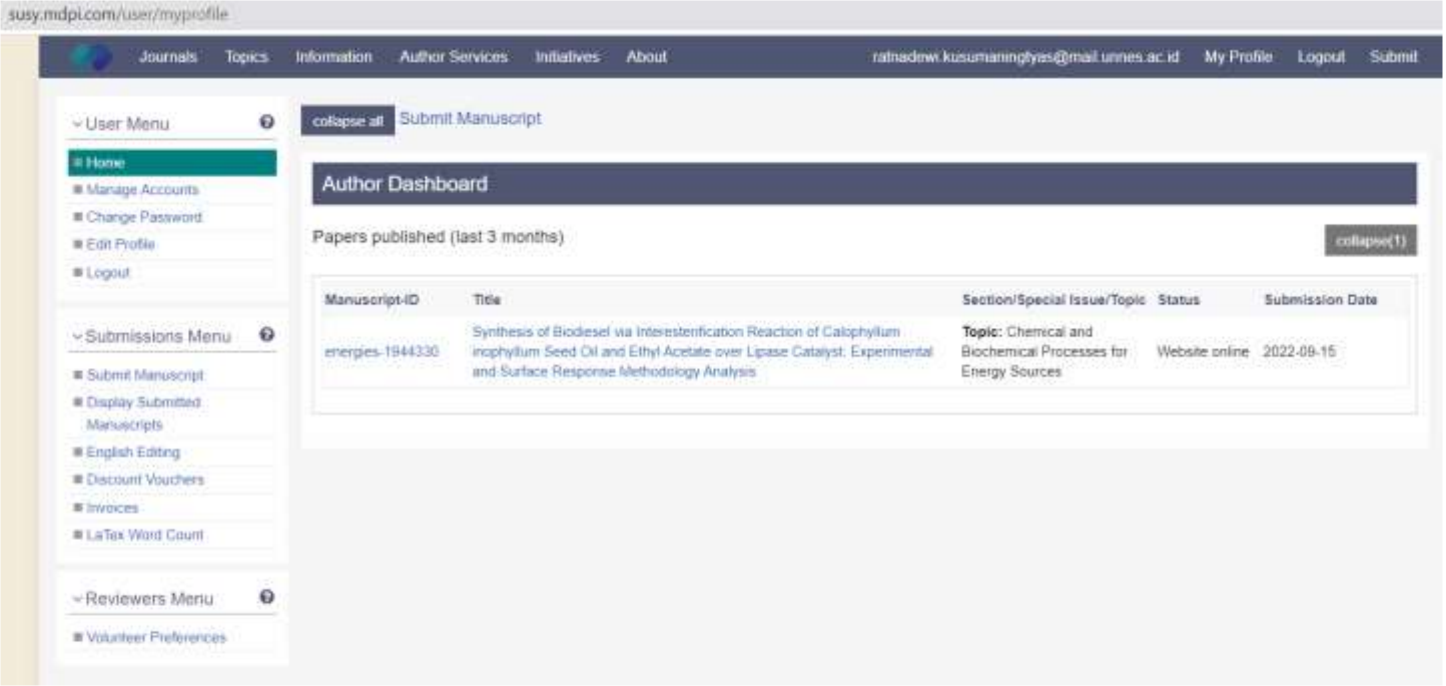

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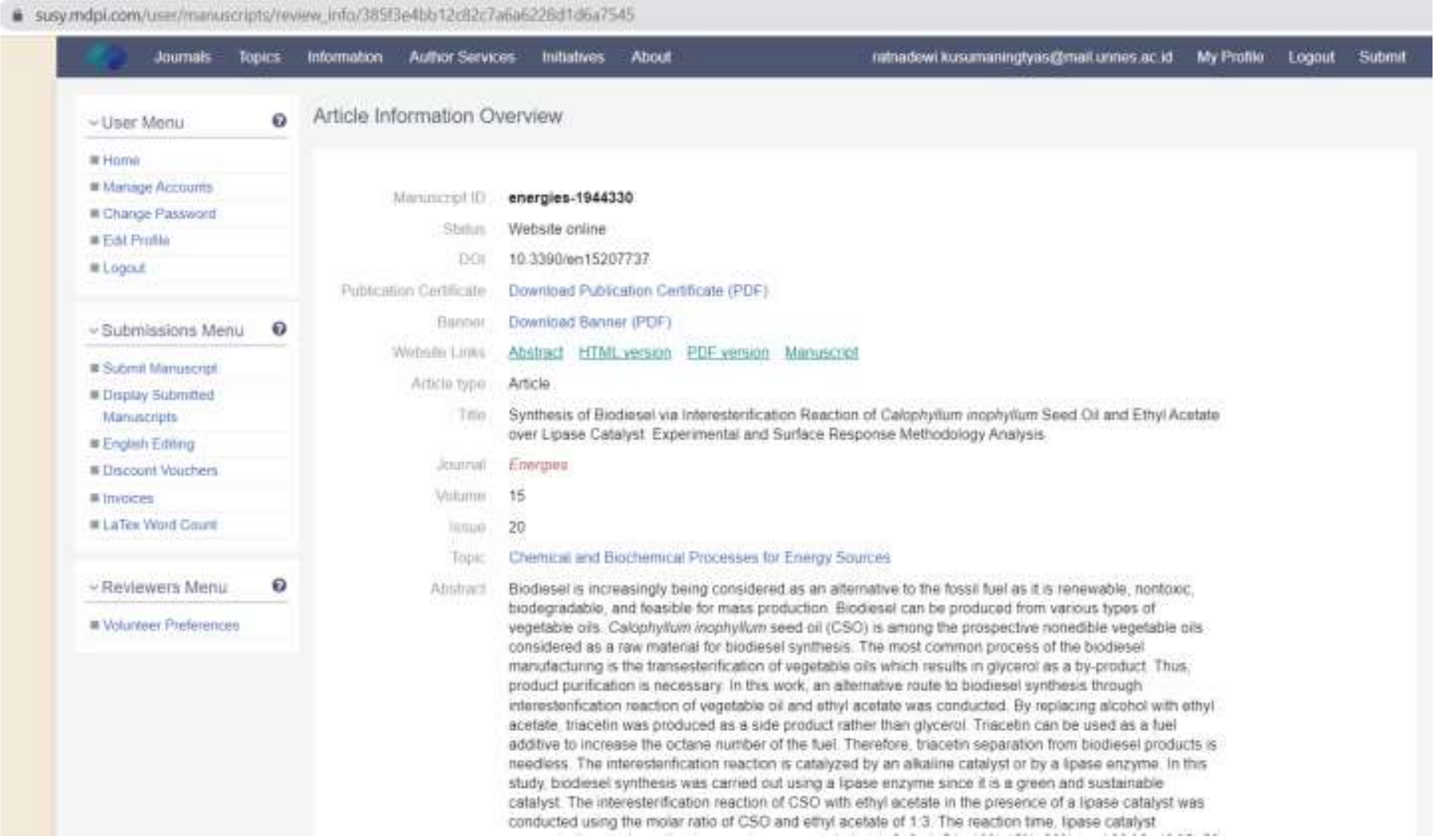

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Journal	*Energies*
Volume	15
Issue	20
Topic	Chemical and Biochemical Processes for Energy Sources
Abstract	Biodiesel is increasingly being considered as an alternative to the fossil fuel as it is renewable, nontoxic, biodegradable, and feasible for mass production. Biodiesel can be produced from various types of vegetable oils. *Calophyllum inophyllum* seed oil (CSO) is among the prospective nonedible vegetable oils considered as a raw material for biodiesel synthesis. The most common process of the biodiesel manufacturing is the transesterification of vegetable oils which results in glycerol as a by-product. Thus, product purification is necessary. In this work, an alternative route to biodiesel synthesis through interesterification reaction of vegetable oil and ethyl acetate was conducted. By replacing alcohol with ethyl acetate, triacetin was produced as a side product rather than glycerol. Triacetin can be used as a fuel additive to increase the octane number of the fuel. Therefore, triacetin separation from biodiesel products is needless. The interesterification reaction is catalyzed by an alkaline catalyst or by a lipase enzyme. In this study, biodiesel synthesis was carried out using a lipase enzyme since it is a green and sustainable catalyst. The interesterification reaction of CSO with ethyl acetate in the presence of a lipase catalyst was conducted using the molar ratio of CSO and ethyl acetate of 1:3. The reaction time, lipase catalyst

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Article

Synthesis of Biodiesel via Interesterification Reaction of *Calophyllum inophyllum* Seed Oil and Ethyl Acetate over Lipase Catalyst: Experimental and Surface Response Methodology Analysis

Ratna Dewi Kusumaningtyas [1,*], Normaliza Normaliza [1], Elva Dianis Novia Anisa [1], Haniif Prasetiawan [1], Dhoni Hartanto [1], Harumi Veny [2], Fazlena Hamzah [2], and Miradatul Najwa Muhd Rodhi [2]

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Abstract: Biodiesel has gained a rising consideration as an alternative to the fossil fuel as it is renewable, nontoxic, biodegradable, and feasible for mass production. Biodiesel can be produced from various types of vegetable oils. *Calophyllum inophyllum* seed oil (CSO) is among the prospective non edible vegetable oil as raw material for biodiesel synthesis. The most common process of the biodiesel manufacturing is the transesterification of vegetable oils which results in glycerol as a by-product. Thus, product purification is necessary. In this work, an alternative route of biodiesel synthesis through interesterification reaction of vegetable oil and ethyl acetate was conducted. By replacing alcohol with ethyl acetate, triacetin is produced as side product rather than glycerol. Triacetin has ability as a fuel additive which can increase the octane number of the fuel. Therefore, triacetin separation from biodiesel product is needless. Interesterification reaction is catalyzed by an alkaline catalyst or by lipase enzyme. In this study, biodiesel synthesis was carried out using lipase enzyme since it is a green and sustainable catalyst. The interesterification reaction of CSO with ethyl acetate in the presence of lipase catalyst was conducted using the molar ratio of CSO and ethyl acetate of 1:3. The reaction time, lipase catalyst concentration, and reaction temperature were varied at 1,2,3,4 ,5 hours, 10%,15%, 20%, and 30°C, 40°C, 50°C, 60°C, respectively. The experimental results were also analyzed using Response Surface Methodology (RSM) with the Box Behnken Design (BBD) model on Design Expert software. Data processing using RSM revealed that the optimum conversion and selectivity were 41.46% and 23.31%, respectively, obtained at the temperature reaction of 44.43°C, reaction time of 5 hours, and lipase catalyst concentration of 20%.

Keywords: biodiesel; *Calophyllum inophyllum* seed oil; interesterification; enzymatic, Box Behnken Design

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1. Introduction

Biodiesel is an alternative fuel for diesel engines which is synthesized from vegetable oils or animal fats. Biodiesel is one of the modern bioenergy which has several advantages. It has non-toxic properties, low emission rates and sulfur gas, just to name a few. Thus, biodiesel is a prospective alternative of the environmentally friendly diesel fuel [1]. Biodiesel is commonly derived from vegetable oil which has renewable characteristic. The main components of vegetable oils and animal fats are triglycerides, which can be converted into mono alkyl esters of long chain fatty acid or termed as fatty acid alkyl ester (biodiesel). Various types of vegetable oils can be applied as prospective raw materials for biodiesel preparation [2]. The widely used raw material for biodiesel synthesis in Indonesia is crude palm oil (CPO). CPO is abundantly available since it is produced on a large scale. However, the production of palm oil biodiesel competes with food needs. Therefore, other alternative vegetable oils are needed as raw materials for biodiesel. Non edible oil feedstocks are favorable to ensure the sustainability biodiesel production [3].

One among the potential non edible vegetable oils in Indonesia is *Calopyllum inophyllum* seed oil (CSO). Akram et al. [1] reported that *Calopyllum inophyllum* (tamanu) seed has oil content of 65-75%, which is higher than other nonedible seed oil plants such as jatropha seed oil (27-40%), rubber seed kernel oil (40-50%), and castor oil (45-65%). According to Adenuga et al. [4], CSO biodiesel meets the Australian, ASTM and EN standards. Therefore, CSO is suitable to be used as feed stock in the production of biodiesel. However, CSO contains high gum, thus degumming process is necessary as the pretreatment step of the biodiesel feedstocks [5].

The most general method for making biodiesel is an alkaline catalyzed transesterification reaction by reacting vegetable oils with short chain alcohols such as methanol or ethanol [6,7]. However, transesterification reaction has a limitation in term of the potential for a saponification side reaction if the oil feedstock contains high free fatty acid (FFA) as described by Kusumaningtyas et al. [8]. Besides, transesterification reaction results in glycerol as by product which needs a separation process to obtain high purity biodiesel. Biodiesel purification from glycerol by-products requires a series of separation steps which brings in high operation cost. To overcome these problems, it is necessary to apply the process of producing biodiesel which is glycerol-free through an interesterification reaction [9]. In the interesterification reaction, the use of methanol or ethanol is replaced with the methyl acetate or ethyl acetate. Interesterification of triglyceride yields triacetin co-product instead of glycerol [10]. Triacetin has advantage as a fuel additive, a good anti knocking and can enhance the octane number. The addition of triacetin to the fuel will also offer an environmental benefit since it reduces the exhaust smoke and NO emissions to some extent. It can be stated that the existence of triacetin co-product in biodiesel will contribute to the engine performance improvement [11]. It occurs since the addition of triacetin increases the oxygen content of the fuel [12]. Thus, it is unnecessary to separate triacetin from biodiesel products.

To date, development of green energy through environmentally friendly processes becomes priority. One alternative towards green process is by reducing the use of chemical catalysts and switching to enzymatic catalyst (biocatalysts). The enzyme catalyzed biodiesel synthesis shows superiority compared to the chemically catalyzed one, mainly in terms of the lower energy requirement and natural conservation aspect. Lipase is the most common enzyme employed for biodiesel synthesis. There are two principal classes of lipase, free enzyme and immobilized enzyme. Free lipase enzyme is cheaper and simpler, thus it is feasible for large scale application [13]. Besides that, the use of lipase enzymes is very promising to overcome the disadvantages of alkaline catalysts related to occurrence of the undesired saponification reaction when the high FFA oil used as raw material. Lipase enzyme catalyst can be applied for the high FFA feedstock without any necessity of FFA removal as the pretreatment step. However, lipase biocatalysts are easily deactivated in an alcoholic environment since short-chain alcohols often cause irreversible loss of enzyme activity [14]. Therefore, the non-alcoholic interesterification route is preferable in order to maintain high biocatalyst activity and stability during the reaction. In the interesterification reaction, alcohol can be replaced by methyl acetate or ethyl acetate which is harmless for the lipase catalyst [15].

In this work, biodiesel was synthesized through lipase catalyzed interesterification of *Calopyllum inophyllum* seed oil (CSO) with ethyl acetate. The lipase enzyme used was the liquid free lipase from Novozyme. The effects of main parameter on the reaction conversion were studied experimentally. Process optimization was also conducted using Response Surface Methodology. Response Surface Methodology (RSM) is a collection of mathematical and statistical calculation techniques based on the compatibility of the empirical model with the experimental data obtained. RSM is useful for modeling and analysis of the problems that influences the response variables with the purpose of optimizing the response. Response Surface Methodology (RSM) analysis is useful in determining the operating conditions to achieve the desired conversion [16]. There are several experimental design models used in RSM, such as Box-Behnken Design (BBD), Control Composite Design (CCD) and Doehlert Design. In this study, optimization of the interesterification reaction of CSO in the presence of lipase enzyme catalyst was performed using BBD in RSM analysis to determine the best reaction operation condition which resulted in the highest reaction conversion.

2. Materials and Methods

2.1. Materials

The materials used were: *Calophyllum inophyllum* seed oil (CSO) from UMKM Samtamanu Cilacap, Indonesia, ethyl acetate p.a (Merck), liquid lipase catalyst (Eversa Transform 2.0 from Novozyme*)*, phosphoric acid p.a (Merck), KOH p.a (Merck), oxalic acid p.a (Merck), ethanol p.a (Merck), and aquadest from Indrasari chemicals store, Semarang, Indonesia.

2.2. Method

2.2.1. Degumming

Crude CSO as much as 100 mL was put into a 250 mL flat bottom flask. The oil was then heated using hot plate equipped with a magnetic stirrer at a speed of 600 rpm until the temperature reached 70°C. Phosphoric acid was subsequently added to the hot oil with the amount of 0.3% (w/w) of CSO and heated for 25 minutes. The oil was cooled at room temperature and put into a 500 mL separatory funnel. Distilled water at a temperature of 40°C was thereafter added with a volume of 5% of the CSO volume. Let the mixture in the separatory funnel for to attain the completion of the separation between the oil and the residue. The degummed CSO was then separated from the residue and was heated in the oven with a temperature of 105°C to reduce the water content [16]. CSO was then analyzed to determine its physical properties (density, viscosity, and acid number).

2.2.2. Interesterification Reaction

After the degumming process, CSO was used as the raw material for biodiesel synthesis via interesterification reaction. CSO as much as 100 mL was introduced into a 250 mL flat bottom three neck flask and added with 10% (w/w) liquid lipase. The mixture was heated using a hot plate equipped with a magnetic stirrer with the stirring speed of 600 rpm. The reaction temperature was varied at 30°C, 40 °C, 50°C and 60°C. After the specified temperature was reached, the ethyl acetate with the similar temperature was added with the ratio of CSO to ethyl acetate of 1:3 (stoichiometric ratio). The reaction time was 1-5 h. Sample was taken periodically every 1 hour of the reaction time. Prior to the analysis, sample was centrifuged with the centrifugation speed of 40,000 rpm for 15 minutes to separate between biodiesel product and the catalyst residue. The sample analysis was carried out using Gas Chromatography-Mass Spectroscopy (GC-MS).

2.2.3. Gas Chromatography-Mass Spectroscopy (GC-MS)

Analysis of the fatty acid composition of the biodiesel was determined using a Gas Chromatography-Mass Spectrometer (GC-MS QP2010 SE) with a column flow rate of

1.20 mL/min, an oven column temperature of 65°C, a pressure of 74.5 kPa, and an injecttion temperature of 250°C with split injection mode. The internal standard used was heptadecanoic acid, methyl ester.

2.2.4. Response Surface Methodology

The GC-MS data was utilized to determine the reaction conversion. The result was subsequently analyzed using Response Surface Methodology (RSM) with the Box Behnken Design (BBD) model on Design Expert software to determine the optimum operation condition. The independent variables and the design of the BBD experiment are presented in Table 1.

Table 1. Independent Variables.

Variable	Values
Reaction Time	1,2,3,4, and 5 hours
Reaction Temperature	30°C, 40°C, 50°C, and 60°C
Catalyst Concentration	10%, 15%, and 20%

3. Results and Discussion

3.1. Effects of the Degumming Process on the CSO Properties

Naturally, the crude CSO contains phospholipids, sterols, free fatty acids, waxes, oil-soluble pigments and hydrocarbons. Degumming is the initial and essential refining process of crude vegetable oil to remove phospholipids and gums [17]. To date, there are various methods that can be employed to degum vegetable oil, explicitly water, acid, enzymatic, and membrane degumming techniques. Water degumming and acid degumming is the most used methods for vegetable degumming at industrial scale [18]. Those methods are simple, easy, and low cost. However, water degumming is less effective compared to the acid degumming. Thus, acid degumming method is more advantageous. The most used acid for degumming process are phosphoric or citric acid with the suggested concentration between 0.05 – 2% w/w oil (Costa et al., 2018). In this work, 0.3% phosphoric acid was applied [9,19]. After the degumming process, the black color of the CSO turns into reddish yellow due to the loss of the phospholipids compound in crude CSO. Phospholipids can form dark color in vegetable oil as a result of the autoxidation process of these compounds during storage. Condensation reaction between the amino groups in phospholipids with aldehydes will yield melanophosphatides compound which give dark color to the oil [20]. Thus, removal of phospholipids in CSO will reduce the occurrence of the above mention reaction and diminish the color intensity.

Subsequent to the degumming process, CSO was settled for 24 hours. After the 24 hours decantation process, the two layers were appeared. The top layer of brownish yellow color was the refined CSO, while the dark brown bottom layer was gum, impurities and water which should be separated. Degumming process can improve the properties of CSO. The effect of degumming process on the CSO properties is indicated in Table 2. It was revealed that the density and viscosity of the CSO were slightly decreasing after the degumming process. It occurred to the removal of gum and other impurities. Besides, degumming also lead to the lessening of the acid number of the oil which was attributable to the decrease of the free fatty acid (FFA) existence in the oil during the degumming process. This phenomenon is in line with the data reported by Adekunle et al.[21]. Fatty acid composition of the CSO after undergoing degumming process was presented in the Table 3, based on the interpretation of the chromatogram shown in Figure 1.

Table 2. Effects of Degumming Process on the Properties of CSO.

CSO Properties	Before Degumming	After Degumming
Density (kg/m^3)	941	937
Viscosity (mm^2/s)	63.42	59.73
Acid Number (mg KOH/kg)	64.62	48.24

Table 3. Fatty Acid Composition of the CSO after Degumming Process.

Fatty Acid	Molecular Weight (g/mol)	Area (%)
Palmitic Acid	256.22	7.82
Linoleic Acid	280.45	16.82
Oleic Acid	282.52	26.62
Stearic Acid	284.47	8.86
Arachidic Acid	312.54	0.31

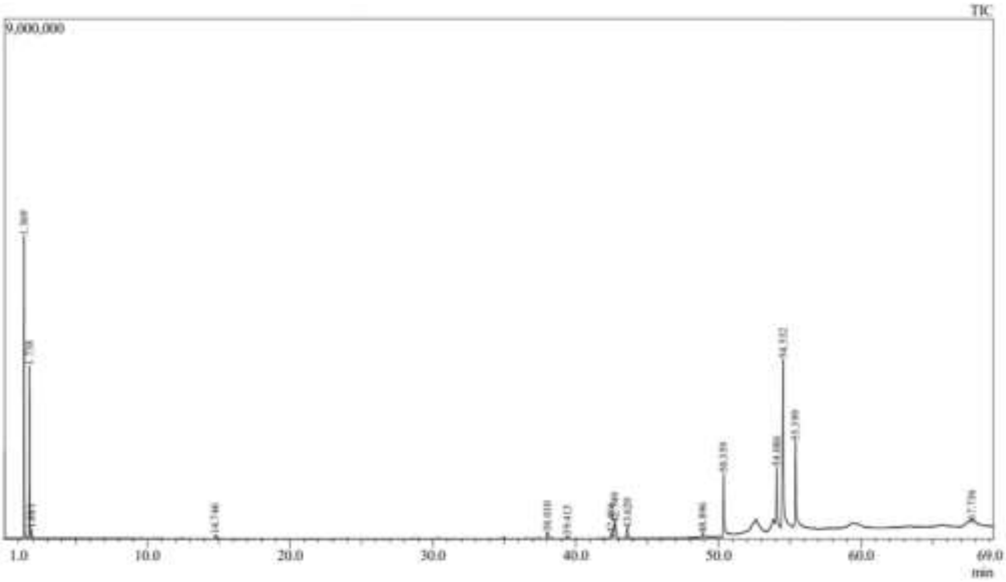


Figure 1. Chromatogram of the CSO after Degumming Process.

3.2. *Effects of the Temperature on the Interesterification Reaction Conversion*

After the degumming process, CSO directly underwent interesterification process with ethyl acetate in the presence of lipase catalyst. A specific pre-treatment for reducing the FFA content of CSO was not considered necessary since the FFA presentation in the oil does not affect the enzymatic catalyzed reaction. The influences of the reaction time and temperature on the reaction conversion were observed at the fixed lipase catalyst concentration of 10% (w/w). The result is exhibited in Figure 2.

Based on the experimental data, it was disclosed that the highest reaction conversion was 54.99%, obtained at the reaction temperature of 40°C and the reaction time of 3 hours. Commonly, the reaction conversion is enhanced with the increasing of the reaction temperature and reaction time since at the higher temperature, the viscosity of the liquid decreases, causing the higher solubility of reactants, mass diffusion, rate of reaction, and conversion [22]. However, in this work, the reaction conversion decreased when the reaction was performed at 50°C for 5 hours. This trend was in accordance with the result recorded by Gusniah et al. [23]. It was reported that the lipase enzyme catalyst activity in the transesterification reaction of waste cooking oil reached the optimum performance at 40 °C and it declined to some extent when the temperature rose to 50 °C. It happened since the lipase catalyst began to denature, causing the damage of the active part of the enzyme at the higher temperature. Beyond the optimum temperature, lipase catalyst becomes unstable and misplaces its tertiary structure which causes the shortfall of its activity [22]. It thus leads to the declining of the reaction rate and conversion. Generally, the lipase enzyme reached the highest activity at the temperature of 40°C – 45°C as stated by Murtius et al. [24], Ayinla et al. [25], and Yazid [26].

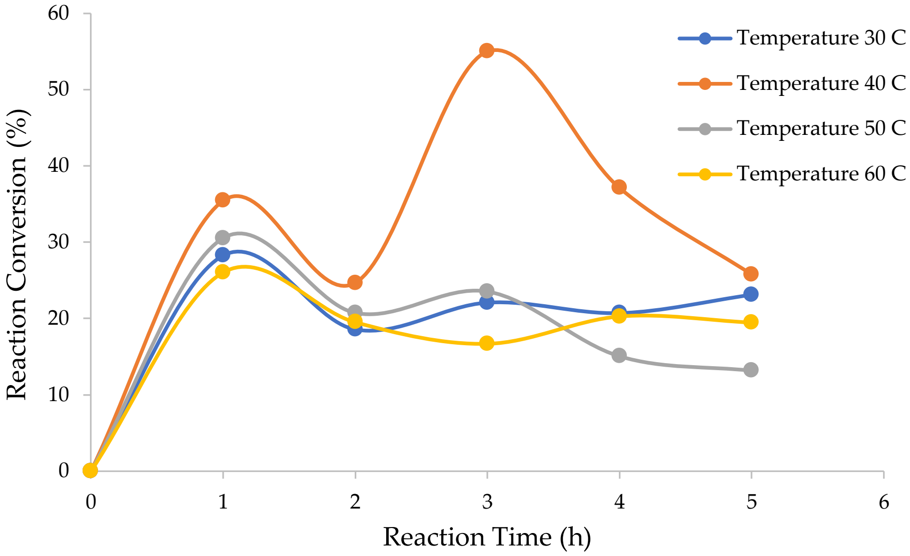


Figure 2. Effects of the reaction time and temperature on the reaction conversion at fixed lipase catalyst concentration of 10% (w/w).

3.3. Effects of the Catalyst Concentration e on the Interesterification Reaction Conversion

Investigation on the influence of the lipase catalyst concentration was carried out at the molar ratio of oil to ethyl acetate of 1:3, reaction temperature of 40°C and reaction time of 5 hours. The lipase concentration studied was 10%, 15%, and 20%. Figure 3 has shown that the optimum catalyst concentration was 15%, resulting in the reaction conversion of 28.445%. This result was in agreement with the resume conveyed by Gusniah et al. [23] which conveyed that the highest lipase catalyst loading was 15%. However, the reaction conversion in this work was still low compared to the other research. It came about since the reaction was conducted with the low molar ratio of oil and ethyl acetate (1:3) and short reaction time (5 h). As comparison, Sun et al. [27] achieved the 94.2% yield for the transesterification of semen Abutili seed oil in the presence of a similar lipase catalyst but at the far excess molar ratio of oil and alcohol (1:7) and longer reaction time (11 h).

Subhedar and Gogate [15] carried out the interesterification reaction of waste cooking oil and methyl acetate using immobilized lipase obtained from *Thermomyces lanuginosus* (Lipozyme TLIM) as a catalyst. The optimum were demonstrated at the oil : methyl acetate molar ratio of 1:12, enzyme concentration of 6% (w/v), reaction temperature of 40 °C and reaction time of 24 h. Biodiesel yield was 90.1% under these operation conditions, which was higher than this work. The higher reaction conversion can be provided since the reaction was conducted on an excessive molar ratio and extensively longer reaction time (24 h). Besides, this reaction employed an immobilized lipase, in which the lipase was attached on the carrier substrate, which led to the higher product purity. Thus, it can be deduced that the lipase catalyzed reaction this work resulted in a lower conversion due to a shorter reaction time and low molar ratio of the reactants, which was not excess than the stoichiometric ratio. Besides that, utilization of the liquid lipase can reduce the apparent concentration of biodiesel yield since it can act as an impurity in the final biodiesel product.

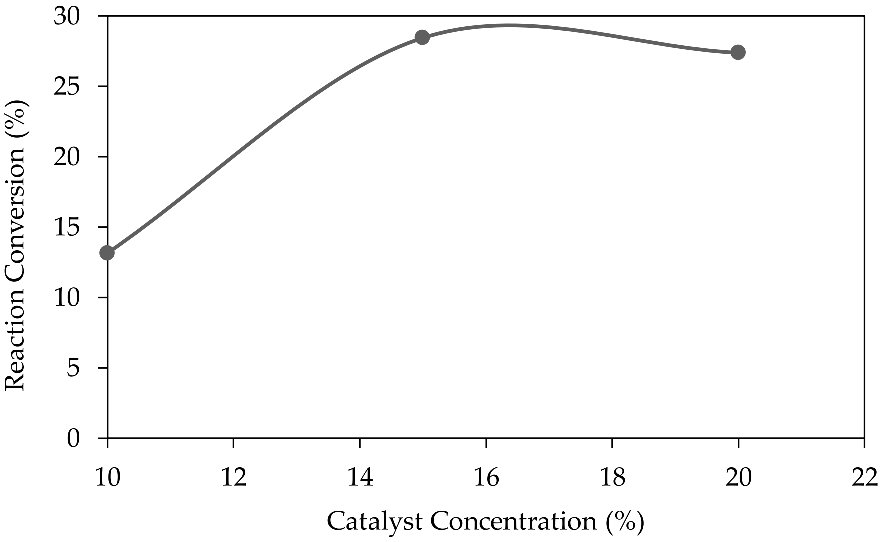


Figure 3. Effects of the Lipase Catalyst Concentration at the Reaction Temperature of 40°C and Reaction Time of 5 Hours.

3.4. Response Surface Methodology (RSM) Analysis

Response surface methodology (RSM) is the broadest employed method of experimental design for optimization. This technique applies mathematical and statistical method for modeling analyzing a process which involves numerous. The purpose of the RSM is to optimize the response variables [28]. The factors that influence the process are entitled dependent variables. Meanwhile, the responses are named as dependent variables. Therefore, analysis using Response Surface Methodology (RSM) is useful in designing operating conditions to achieve the targeted conversion. Many literatures have shown that the most applicable tools in RSM for optimization of biodiesel production process are the Box Behnken Design (BBD) and Central Composite Design (CCD) tools [29]. BBD is favored than CCD since it provides higher efficiency but more economical [30,31]. To evade the failure and extreme reaction variables value, BBD is limited to three levels. Hence, BBD typically has no factorial points. The BBD tool was also utilized by Sharma et al. [32] and Rokni et al. [33].

In this study, BBD was applied to obtain the optimal operating conditions for biodiesel synthesis via interesterification reaction. The Box Behnken Design (BBD) tool in RSM was used to evaluate the effect of the independent variables (reaction time, reaction temperature, and catalyst concentration). The experimental data of the reaction conversion compared with the prediction using BBD is exhibited in Table 4.

The parameters used to obtain the appropriate model to predict the response results must match the observed model to the experimental data. Analysis of variance (ANOVA) was accomplished using the most common types of polynomial models, namely linear, interactive (2FI), quadratic and cubic can be applied to predict the response variables of experimental data. Several parameters such as sequential p-value, lack of fit p-value, adjusted R¬2, predicted R2 and Adiq precision were used to conclude the most suitable type of model for the optimization of the conversion of biodiesel produced from CSO. In this study, optimization using RSM was initially performed using the quadratic model. The ANOVA quadratic model is presented in Table 5.

Table 4. Experimental Data of the Reaction Conversion and the Prediction Using BBD.

Run	Temperature, °C	Reaction Time, hr	Catalyst Concentration, % w/w	Reaction Conversion, % Experiment	Prediction	Error, %
1	40	3	15	12.92	18.15	40.4
2	30	3	10	22.08	19.877	9.97
3	40	5	20	48.92	48.03	1.8
4	30	3	20	27.75	27.33	1,5
5	40	5	10	25.77	26.66	3.4
6	40	3	15	18.27	18.15	0.6
7	30	1	15	23.41	24.72	5.6
8	50	5	15	14.90	13.59	8.8
9	40	1	20	13.55	12,66	6.5
10	30	5	15	22.10	23.41	5.9
11	40	3	15	23.25	18.15	21.9
12	40	1	10	35.43	36.32	2.5
13	50	1	15	26.82	25.51	4.8
14	50	3	20	12.01	14.22	18.3
15	50	3	10	23.53	23.96	1.7

Table 5. ANOVA of the Quadratic Model.

Source	Sum of Squares	DF	Mean Square	F-value	p-value	
Model	1007.96	9	112.00	2.13	0.2098	not significant
A-Suhu	40.83	1	40.83	0.7760	0.4187	
B-Waktu	19.45	1	19.45	0.3696	0.5698	
C-Katalis	2.61	1	2.61	0.0497	0.8325	
AB	28.14	1	28.14	0.5348	0.4974	
AC	73.90	1	73.90	1.40	0.2892	
BC	507.01	1	507.01	9.64	0.0267	
A^2	32.27	1	32.27	0.6134	0.4690	
B^2	161.73	1	161.73	3.07	0.1399	
C^2	139.90	1	139.90	2.66	0.1639	
Residual	263.06	5	52.61			
Lack of Fit	209.74	3	69.91	2.62	0.2881	not significant
Pure Error	53.32	2	26.66			
Cor Total	1271.02	14				

Based on the quadratic model, it was revealed that the p-value was 0.2998, which did not meet the requirement of the p-value < 0.05 as demonstrated in Tabel 5. Hence, the quadratic model was not significant for this case. To overcome this obstacle, modified model was developed to obtain the significant model [34]. Modification of the model shown that the reduced cubic model made evident of the significant model (Table 6).

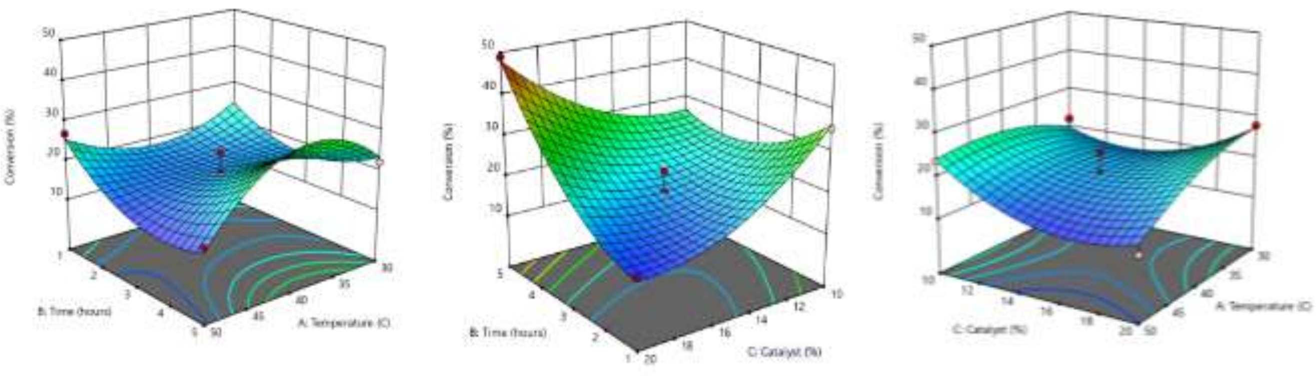
Table 6. ANOVA of the Reduced Cubic Model.

Source	Sum of Squares	DF	Mean Square	F-value	p-value	
Model	1197.58	10	119.76	6.52	0.0428	significant
A-Suhu	40.83	1	40.83	2.22	0.2102	
B-Waktu	165.26	1	165.26	9.00	0.0399	
C-Katalis	2.61	1	2.61	0.1423	0.7252	
AB	28.14	1	28.14	1.53	0.2834	
AC	73.90	1	73.90	4.03	0.1153	
BC	507.01	1	507.01	27.62	0.0063	
A^2	32.27	1	32.27	1.76	0.2555	
B^2	161.73	1	161.73	8.81	0.0412	
C^2	139.90	1	139.90	7.62	0.0508	
A^2B	189.62	1	189.62	10.33	0.0325	
Residual	73.44	4	18.36			
Lack of Fit	20.12	2	10.06	0.3773	0.7260	not significant
Pure Error	53.32	2	26.66			
Cor Total	1271.02	14				
Adeq Precision	9.6401					

It was found that the p-value of the modified cubic model was 0.0428, which has met the requirement of p-value > 0.05. Furthermore, the value of lack-of-fit was examined. Lack-of-fit is a test that analyzes how satisfactory the full models suit with the data. Model with a significant lack-of-fit should not be applied for the prediction. In this work, the lack-of-fit was not significant, which means that the model can be employed for the prediction. The value of adeq precision is the measure of the ratio of the signal to the disturbance. The expected value of the ratio is > 4 [29]. This model provided the value of the Adeq precision of 9.6401, indicating that the model is proper. Therefore, the reduced cubic model can be used to describe the response on the reaction conversion. The empirical correlation of the reduced cubic model in the form of polynomial orde for the reaction conversion in the CSO esterification is displayed in Equation 1.

$$\text{Conversion (\%)} = 18.15 - 2.26\,A + 6.43\,B - 0{,}5715\,C - 2.65\,AB - 4.30\,AC + 11.26\,BC - 2.96\,A^2 + 6.62\,B^2 + 6.16\,C^2 - 9.74\,A^2B \tag{1}$$

Where, A is the reaction temperature (°C), B is the reaction time (min) and C is the Catalyst Concentration (%). Based on the reduced cubic model, analysis on the effects and the process variables interaction on the response variable were carried out using 3D RSM graphs. The results are presented in Figure 4.



(a) (b) (c)

Figure 4. The 3D RSM graph representing the effects of reaction time, temperature and catalyst concentration on the reaction conversion.

Figure 4(a) shows the interaction between temperature and reaction time which gives a significant change to the increase in conversion. It was found that there was an increase in conversion at the temperature of 40°C and reaction time of 5 hours. This phenomenon is in line with the investigation of Handayani et al. [34], which reported that the optimum conversion was obtained at the temperature of 45°C and reaction time of 6 hours. Figure 4(b) discloses that the longer the reaction time employed, the higher the conversion achieved. It happened since the reaction time is directly proportional to the amount of product yielded. Li et al. [35] conveyed that the reaction conversion of *Pseudomonas cepacia* lipase was low at the reaction time of 3 h. (44%). On the contrary, it rose to 73% when the reaction time extended to 12 h. Figure 4(c) bares that the lower the reaction temperature applied, the higher conversion resulted in. It occurred since the lipase enzyme activity works at the temperature of 30°C – 45°C. In the enzymatic reaction, the increase of the temperature will promote the reaction rate and accordingly the reaction conversion due to the decrease of the viscosity which enhance the solubility of oil and methanol. However, at a certain temperature, reaction conversion declines evoking the enzyme deactivation [36]. Meanwhile, the addition of the catalyst concentration increased the reaction conversion. This result was in line with the description of Gusniah et al. [23]. It was justified that the increasing amount of the enzyme employed will provide a higher amount of the available active sites for the reaction, leading to the higher reaction conversion.

The Derringer method is utilized to optimize the response which is characterized by the presence of a desirability function. Desirability shows how close the optimization results are to the optimum point. The desirability function (DF) is constructed on the transformation of all the acquired responses from different scales into a scale-free value. The values of DF are ranging from 0 to 1. The value 0 means that the factors provide an undesirable response. On the other hand, the value 1 denotes the optimal performance of the evaluated factor [37]. Hence, the desirability value which is close to 1 is the expected value. However, the RSM analysis is not only to find the desirability value which is equal to 1, but to determine the conditions that match expectations. The multiple correlation coefficient (R^2) was closed to 1. It denoted that the models have good predictive ability. Based on the optimization results, the optimum conversion was 41.46%, attained at a temperature of 44.43°C, a reaction time of 5 hours and a catalyst concentration of 20%, with a desirability value of 0.733.

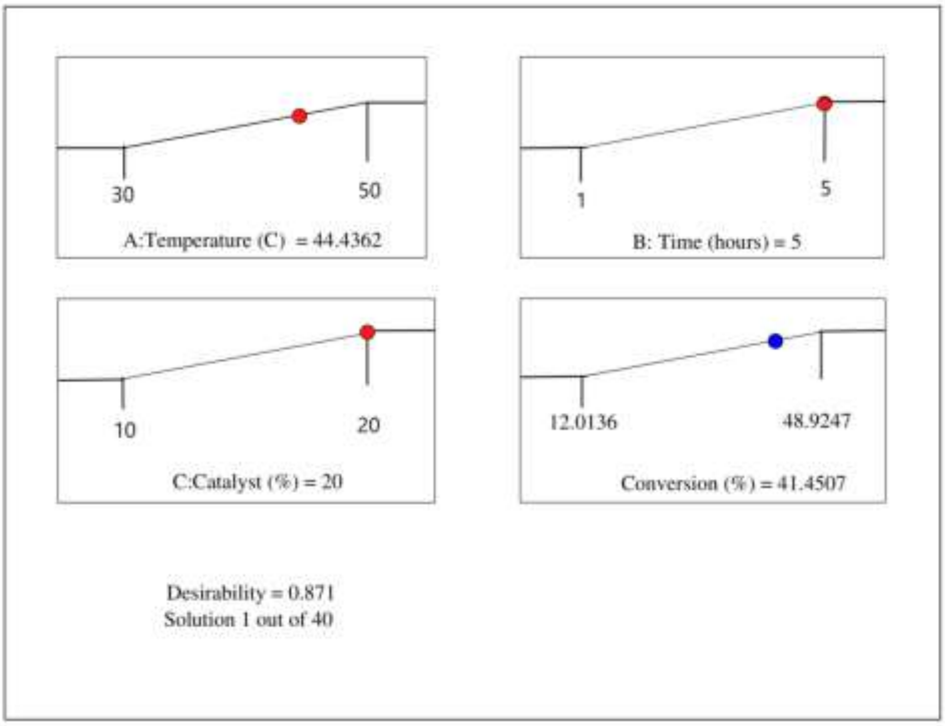


Figure 7. Reaction Conversion Optimization Using RSM

5. Conclusions

Based on the RSM analysis, it was revealed that reaction time and catalyst concentration had significant effects on the conversion of the interesterification reaction of CSO with ethyl acetate in the presence of lipase catalyst for biodiesel synthesis. The results of the analysis showed that the optimum conversion was 41.46% at the reaction temperature, reaction time, and catalyst concentration of 44.40°C, 5 hours, and 20%, respectively.

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Dear Dr. Kusumaningtyas,

Thank you again for your manuscript submission:

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Type of manuscript: Article
Title: Synthesis of Biodiesel via Interesterification Reaction of Calophyllum inophyllum Seed Oil and Ethyl Acetate over Lipase Catalyst: Experimental and Surface Response Methodology Analysis
Authors: Ratna Dewi Kusumaningtyas *, Normaliza Normaliza, Elva Dianis Novia Anisa, Haniif Prasetiawan, Dhoni Hartanto, Harumi Veny, Fazlena Hamzah, Miradatul Najwa Muhd Rodhi
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Manuscript ID	**energies-1944330**
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Article type	Article
Title	Synthesis of Biodiesel via Interesterification Reaction of Calophyllum inophyllum Seed Oil and Ethyl Acetate over Lipase Catalyst: Experimental and Surface Response Methodology Analysis
Journal	*Energies* (https://www.mdpi.com/journal/energies)
Topic	Chemical and Biochemical Processes for Energy Sources (https://www.mdpi.com/topics/chemical)
Abstract	Biodiesel has gained a rising consideration as an alternative to the fossil fuel as it is renewable, nontoxic, biodegradable, and feasible for mass production. Biodiesel can be produced from various types of vegetable oils. Calophyllum inophyllum seed oil (CSO) is among the prospective non edible vegetable oil as raw material for biodiesel synthesis. The most common process of the biodiesel manufacturing is the transesterification of vegetable oils which results in glycerol as a by-product. Thus, product purification is necessary. In this work, an alternative route of biodiesel synthesis through interesterification reaction of vegetable oil and ethyl acetate was conducted. By replacing alcohol with ethyl acetate, triacetin is produced as side product rather than glycerol. Triacetin has ability as a fuel additive which can increase the octane number of the fuel. Therefore, triacetin separation from biodiesel product is needless. Interesterification reaction is catalyzed by an alkaline catalyst or by lipase enzyme. In this study, biodiesel synthesis was carried out using lipase enzyme since it is a green and sustainable catalyst. The interesterification reaction of CSO with ethyl acetate in the presence of lipase catalyst was conducted using the molar ratio of CSO and ethyl acetate of 1:3. The reaction time, lipase catalyst concentration, and reaction temperature were varied at 1,2,3,4 ,5 hours, 10%,15%, 20%, and 30°C, 40°C, 50°C, 60°C, respectively. The experimental results were also analyzed using Response Surface Methodology (RSM) with the Box Behnken Design (BBD) model on Design Expert software. Data processing using RSM revealed that the optimum conversion and selectivity were 41.46% and 23.31%, respectively, obtained at the temperature reaction of 44.43°C, reaction time of 5 hours, and lipase catalyst concentration of 20%.
Keywords	biodiesel; Calophyllum inophyllum seed oil; interesterification; enzymatic, Box Behnken Design

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Journal	Energies (https://www.mdpi.com/journal/energies) (ISSN 1996-1073)
Manuscript ID	energies-1944330
Type	Article
Title	Synthesis of Biodiesel via Interesterification Reaction of Calophyllum inophyllum Seed Oil and Ethyl Acetate over Lipase Catalyst: Experimental and Surface Response Methodology Analysis
Authors	Ratna Dewi Kusumaningtyas * , Normaliza Normaliza , Elva Dianis Novia Anisa , Haniif Prasetiawan , Dhoni Hartanto , Harumi Veny , Fazlena Hamzah , Miradatul Najwa Muhd Rodhi
Topic	Chemical and Biochemical Processes for Energy Sources (https://www.mdpi.com/topics/chemical)
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Authors' Responses to Reviewer's Comments (Reviewer 1)

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Comments and Suggestions for Authors

This is a manuscript that correctly addresses the possibility of obtaining technically and economically viable biofuels, to contribute to the gradual substitution of fossil fuels.Thus, it proposes the use of a non-edible vegetable oil as raw material for the synthesis of the biofuel. This avoids the potential competition of the biofuel with the food uses of the vegetable oil used as feedstock. In addition, a process is being investigated that, together with the mixtures of FAEEs, it is generated some derivatives of glycerol (the corresponding ,mono., di- and triacetates), all they soluble in the fuel itself. This avoids the main drawback of conventional biodiesel, which is the production of high amounts of glycerol (> 15%) that are difficult to manage, and that act in practice as a waste.
The experimental work, impeccably carried out by enzyme

The experimental work, impeccably carried out by enzyme catalysis and Analyzed by using Response Surface Methodology (RSM) accurately determines the optimum operating conditions as well as the foreseeable yields.Therefore, this manuscript is of great interest for its publication, because it represents a model of research line through which the large amount of research on biodiesel must be redirected, and it can be accepted in its present format.

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Author's Reply to the Review Report (Reviewer 2)

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* Author's Notes to Reviewer

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(x) I don't feel qualified to judge about the English language and style

	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	(x)	( )	( )	( )
Are all the cited references relevant to the research?	(x)	( )	( )	( )
Is the research design appropriate?	( )	(x)	( )	( )
Are the methods adequately described?	( )	(x)	( )	( )
Are the results clearly presented?	( )	(x)	( )	( )

Are the conclusions supported by the results?	( )	(x)	( )	( )

Comments and Suggestions for Authors	I believe the authors can not mention that they achieved optimization owing low conversion values obtained. In fact in its response they said "We believe that this work will give valuable contribution for the future work in the area of green technology for biodiesel production". Please change the optimization focus.
Submission Date	15 September 2022
Date of this review	05 Oct 2022 15:28:50

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Reviewers Menu

- Volunteer Preferences

Journal	Energies (https://www.mdpi.com/journal/energies) (ISSN 1996-1073)
Manuscript ID	energies-1944330
Type	Article
Title	Synthesis of Biodiesel via Interesterification Reaction of Calophyllum inophyllum Seed Oil and Ethyl Acetate over Lipase Catalyst: Experimental and Surface Response Methodology Analysis
Authors	Ratna Dewi Kusumaningtyas * , Normaliza Normaliza , Elva Dianis Novia Anisa , Haniif Prasetiawan , Dhoni Hartanto , Harumi Veny , Fazlena Hamzah , Miradatul Najwa Muhd Rodhi
Topic	Chemical and Biochemical Processes for Energy Sources (https://www.mdpi.com/topics/chemical)
Abstract	Biodiesel has gained a rising consideration as an alternative to the fossil fuel as it is renewable, nontoxic, biodegradable, and feasible for mass production. Biodiesel can be produced from various types of vegetable oils. Calophyllum inophyllum seed oil (CSO) is among the prospective non edible vegetable oil as raw material for biodiesel synthesis. The most common process of the biodiesel manufacturing is the transesterification of vegetable oils which results in glycerol as a by-product. Thus, product purification is necessary. In this work, an alternative route of biodiesel synthesis through interesterification reaction of vegetable oil and ethyl acetate was conducted. By replacing alcohol with ethyl acetate, triacetin is produced as side product rather than glycerol. Triacetin has ability as a fuel additive which can increase the octane number of the fuel. Therefore, triacetin separation from biodiesel product is needless. Interesterification reaction is catalyzed by an alkaline catalyst or by lipase enzyme. In this study, biodiesel synthesis was carried out using lipase enzyme since it is a green and sustainable catalyst. The interesterification reaction of CSO with ethyl acetate in the presence of lipase catalyst was conducted using the molar ratio of CSO and ethyl acetate of 1:3. The reaction time, lipase catalyst concentration, and reaction temperature were varied at 1,2,3,4 ,5 hours, 10%,15%, 20%, and 30°C, 40°C, 50°C, 60°C, respectively. The experimental results were also analyzed using Response Surface Methodology (RSM) with the Box Behnken Design (BBD) model on Design Expert software. Data processing using RSM revealed that the optimum conversion and selectivity were 41.46% and 23.31%, respectively, obtained at the temperature reaction of 44.43°C, reaction time of 5 hours, and lipase catalyst concentration of 20%.

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Authors' Responses to Reviewer's Comments (Reviewer 3)

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English language and style	( ) Extensive editing of English language and style required ( ) Moderate English changes required ( ) English language and style are fine/minor spell check required (x) I don't feel qualified to judge about the English language and style

	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	(x)	( )	( )	( )
Are all the cited references relevant to the research?	(x)	( )	( )	( )
Is the research design appropriate?	( )	(x)	( )	( )
Are the methods adequately described?	( )	( )	(x)	( )
Are the results clearly presented?	( )	(x)	( )	( )
Are the conclusions supported by the results?	( )	(x)	( )	( )

Comments and Suggestions for Authors

In the introduction, it would be useful to report some examples of non-edible vegetable oils already widely used as feedstock for biodiesel production.

Row 53: Specify what is the triglyceride and fatty acid content (possibly also what type) in the plant Calopyllum inophyllum.

Row 57-59: What is the gum content? At the industrial level, does the gum content particularly affect the economic viability of using this plant, considering that it requires gum removal as an obligatory step? Is the gum removal step obligatory or does it serve solely to increase interesterification yields? Insert this information in the text.

In materials and methods, insert some more information about the enzyme used. What is actually the temperature optimum of the enzyme used? And what is the tolerance range? Insert this information in the text.

Row 138-139: How come 1:3 was chosen as the molar ratio? is a rather low ratio. It is known from literature that increasing this ratio (with limits) increases the rate of reaction, being then able to recover the alcohol in access. Also, from what time are considered 1,2,3,4,5 h? from the mixing of the reactants or from the reaching of temperature? Was the temperature reached gradually?

Were biodiesel quality standards taken into account in the experimental design? If yes according to what standard?

Row 166-167: Delete the sentence "However, water degumming is less effective than acid degumming," as it is repetitive.

Row 177-180: This part would be better put in materials and methods since in results and discussion the focus on the results obtained is lost.

Row 182-183: Delete the sentence "decreasing after the 182 degumming process. It occurred to the removal of gum and other impurities." as it is repetitive.

Row 196-198: To exclude pretreatment, did you determine the free fatty acid content or rely on literature data? In either case, specify the percentage of free fatty acids.

Row 210: Knowing already from literature that at 50°C the enzyme is denatured why did you test 60°C?

Row 226: Delete the sentence "which conveyed that the highest lipase catalyst loading was 15%."

Conclusions: Expand on the conclusions. Given the process costs and the need for biodiesel purification, can the 41.46 % yield be considered advantageous? Can the process be applied on an industrial scale, or is optimization required to be effectively applicable?

Submission Date	15 September 2022
Date of this review	21 Sep 2022 16:09:33

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Authors	Ratna Dewi Kusumaningtyas * , Normaliza Normaliza , Elva Dianis Novia Anisa , Haniif Prasetiawan , Dhoni Hartanto , Harumi Veny , Fazlena Hamzah , Miradatul Najwa Muhd Rodhi
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Authors' Responses to Reviewer's Comments (Reviewer 4)

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( ) I don't feel qualified to judge about the English language and style

	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	(x)	( )	( )	( )
Are all the cited references relevant to the research?	(x)	( )	( )	( )
Is the research design appropriate?	( )	( )	(x)	( )
Are the methods adequately described?	( )	(x)	( )	( )
Are the results clearly presented?	( )	( )	(x)	( )
Are the conclusions supported by the results?	( )	( )	(x)	( )

Comments and Suggestions for Authors

1. The Introduction could benefit from a scheme elucidating about the processes being discussed: the common transesterification and the interesterification proposed here.

2. In the Experimental Section a reference for the Design Expert software is missing.

3. The conversion in Figure 3 for the catalyst loading of 10% was ca. 14% at 40ºC and after 5h. Why is this result different from that reported in Figure 2, almost 2x, for the same conditions? And why have all the temperatures and reaction times been tested? At least I would expect the best condition from Figure 2 to be tested (40 ºC and 3h). Authors must address this.

4. Again the data in Table 4 doesn't correlate with Figure 2, Please check and correct.

5. Concerning data in Figure 7 showing the optimized reaction parameters. What I do not understand is that is how those parameters are worse than the data showing in Figure 3 for the reaction at 40 ºC after 3h and 10% catalyst, with conversion at almost 60%. Explanation must be provided.

Submission Date	15 September 2022
Date of this review	26 Sep 2022 13:07:29

RESPONSE TO REVIEWER

REVIEWER 1

No	Reviewer Comment	Response
1	This is a manuscript that correctly addresses the possibility of obtaining technically and economically viable biofuels, to contribute to the gradual substitution of fossil fuels. Thus, it proposes the use of a non-edible vegetable oil as raw material for the synthesis of the biofuel. This avoids the potential competition of the biofuel with the food uses of the vegetable oil used as feedstock. In addition, a process is being investigated that, together with the mixtures of FAEEs, it is generated some derivatives of glycerol (the corresponding ,mono., di- and triacetates), all they soluble in the fuel itself. This avoids the main drawback of conventional biodiesel, which is the production of high amounts of glycerol (> 15%) that are difficult to manage, and that act in practice as a waste. The experimental work, impeccably carried out by enzyme catalysis and Analyzed by using Response Surface Methodology (RSM) accurately determines the optimum operating conditions as well as the foreseeable yields. Therefore, this manuscript is of great interest for its publication, because it represents a model of research line through which the large amount of research on biodiesel must be redirected, and it can be accepted in its present format.	Thank you very much for your positive review, and hopefully this work can provide significant contribution towards green renewable energy synthesis.

REVIEWER 2

No	Reviewer Comment	Response
1	The titled work “Synthesis of biodiesel via interesterification reaction of *calophyllum inophyllum* seed oil and ethyl acetate over lipase catalyst: experimental and surface response methodology analysis” was reviewed. The topic is appropriated to Energies; however, there are some issues to attend before the publication. The contribution is good in extension and presentation.	Thank you very much for the review and your encouraging suggestion.
2	I believe that the selection of levels of factors did not allow to estimate the significant effect of all of the factors.	The range of the parameters were set based on the literature review and were at the appropriate levels of factors. The lipase is active in the temperature range of 20°C – 60°C, Samsumaharto (2008) and Thongprajukaew et al. (2010), and mostly the predictive optimum temperature is between 30°C-50°C. The reaction time is often studied up to 12 h, but the significant reaction rate is at 0 – 5 h (Sun et al., 2021 https://doi.org/10.1016/j.indcrop.2021.113643). Catalyst concentration range was set up based on our preliminary study in which the significant conversion obtained when the catalyst loading is not lower than 10%. The molar ratio was fixed at 1:3 since we want to investigate the enzymatic reaction in the absence of excess reactant as it was conducted by Manurung et al. (2015).

No	Reviewer Comment	Response
3	Moreover, the Figure 4 do not show the maxima, or optimal, expected value of conversion.	Actually, the optimization was also combined with the selectivity. However, the highest selectivity is not address in this manuscript and not the focus of this study.
4	In fact, the authors obtained an optimal conversion of 41.46% which is distant of 80 or 90% values.	Based on the investigated range of variables (temperature, catalyst concentration, and reaction time), we found that the optimal conversion was 41.46%. These results can be used as a basis to modify the variables and developing process intensification in further research in order to obtain a higher conversion.
5	I suggest to reject the work for the mentioned reasons.	We believe that this work will give valuable contribution for the future work in the area of green technology for biodiesel production.

REVIEWER 3

No	Reviewer Comment	Response
1	In the introduction, it would be useful to report some examples of non-edible vegetable oils already widely used as feedstock for biodiesel production.	There are several non edible oils that have been widely investigated as biodiesel feedstocks such as silk–cotton or *Ceiba pentandra* seed oil (Soosai et al., 2022), jatropha oil (Chang et al., 2020; Kusumaningtyas et al., 2017), castor oil (Khan et al., 2022), rubber seed oil (Lüneburger et al., 2022), karanja oil (Tasneem et al., 2022), mahua oil, neem oil (Sayyed et al., 2022), waste cooking oil (Gaur & Goyar 2022), palm fatty acid distillate (Buchori et al., 2022), and *Calophyllum inophyllum* seed oil (Adenuga et al., 2021).
2	Row 53: Specify what is the triglyceride and fatty acid content (possibly also what type) in the plant Calopyllum inophyllum.	Rasyid et al. (2018) reported that fatty acids composition of CSO comprises of oleic acid, palmitic acid, linoleic acid, and stearic acid.
3	Row 57-59: What is the gum content? At the industrial level, does the gum content particularly affect the economic viability of using this plant, considering that it requires gum removal as an obligatory step? Is the gum removal step obligatory or does it serve solely to increase interesterification yields? Insert this information in the text.	However, CSO contains high gummy substances, especially in the form of lipoid A (the hydratable phosphatides) and lipoid B (the non-hydratable phosphatides (Kulkarni et al., 2014; Dijkstra 2017), waxes, and other impurities (Dumitru, 2019). The existence of gum is unfavorable since it may lower the reaction rate which in consequence reduces the biodiesel yield. At industrial level, this condition leads to the economic disadvantage. Besides, the presence of phosphorus compounds can generate the carbon particle deposition in diesel engine which makes plugging in the engine filter, line, and injectors as well as reducing the engine performance (Sharma et al., 2019; Dumitru, 2019). Thus, degumming process is essential as the pretreatment step of the biodiesel feedstocks with high phosphorus content.

No	Reviewer Comment	Response
4	In materials and methods, insert some more information about the enzyme used. What is actually the temperature optimum of the enzyme used? And what is the tolerance range? Insert this information in the text.	The catalyst used was liquid (free form) lipase Eversa Transform 2.0 (ET 2.0) from Novozyme, which is made from genetically modifed Aspergillus oryzae microorganism (Remonatto et al., 2022). Commonly, lipase are active in broad range of temperature from 20-60 °C, and the ET 2.0 was reported to work satisfactorily up to the reaction temperature of 60°C (Gutierrez-Lazaro et al., 2018).
5	Row 138-139: How come 1:3 was chosen as the molar ratio? is a rather low ratio. It is known from literature that increasing this ratio (with limits) increases the rate of reaction, being then able to recover the alcohol in access.	1:3 is the stoichiometric ratio of the CSO to ethyl acetate in the esterification reaction. This study would like to investigate the optimum reaction conversion that can be reached using stoichiometric ratio, with no excess of the reactant as it was conducted by Manurung et al. (2015). This statement has also been added in the paragraph.
6	Also, from what time are considered 1,2,3,4,5 h? from the mixing of the reactants or from the reaching of temperature? Was the temperature reached gradually?	Determination of the reaction temperature of 1, 2, 3, 4, 5 h were starting from the mixing of the reactants at the designed operating temperature. The two reactants namely CSO and ethyl acetate were heated separately prior the reaction. CSO which has been mixed with the lipase were heated in the reactor, whereas ethyl acetate was warmed up separately. When they reached the desired reaction temperature (for example 40°C), ethyl acetate was subsequently poured into the reactor and mixed with the CSO and lipase. The incident was recorded as the reaction time of zero (t = 0 h). Hence, the reaction temperature didn't reach gradually. The description in the interesterification method has been revised according to this explanation.
7	Were biodiesel quality standards taken into account in the experimental design? If yes according to what standard?	This work was only focus to study the effects of operating variables to the reaction conversion. The biodiesel quality standards were not taken into account.

No	Reviewer Comment	Response
8	Row 166-167: Delete the sentence "However, water degumming is less effective than acid degumming," as it is repetitive.	The sentences have been refined: **"**However, water degumming is less effective compared to the acid method. Gum comprises of the hydratable (HP) and non-hydratable phosphatides (NHP). HP can be eliminated using water. In contrast, NHP cannot be eradicated via water degumming (Kulkarni et al., 2014; Dijkstra, 2017). Thus, acid degumming method is more advantageous in which it can remove both the HP and NHP."
9	Row 177-180: This part would be better put in materials and methods since in results and discussion the focus on the results obtained is lost.	The sentences have been removed from the result and discussion part, and put in the material and methods (degumming method).
10	Row 182-183: Delete the sentence "decreasing after the 182 degumming process. It occurred to the removal of gum and other impurities." as it is repetitive.	The sentences have been revised to be: **"**It was revealed that the density and viscosity of the CSO were slightly decreasing after the degumming process due to the removal of gum and other impurities"
11	Row 196-198: To exclude pretreatment, did you determine the free fatty acid content or rely on literature data? In either case, specify the percentage of free fatty acids.	The fatty acid content was determined in this work and the result has been shown in Table 2. The sentences have also been revised: "As depicted in Table 2, the crude and degummed CSO contained high FFA of 32.47% and 24.25%, respectively, which were over 2 mgKOH/g. However, enzymatic reaction is generally insensitive to the existence of the FFA impurities in the oil feedstock. The possibility of the undesired saponification reaction can be neglected. Therefore, a specific pre-treatment for reducing the FFA content of CSO is considered unnecessary since the FFA presentation in the oil does not affect the enzymatic catalyzed reaction (Sebastian et al., 2016; Chang et al., 2021)."

No	Reviewer Comment	Response
12	Row 210: Knowing already from literature that at 50°C the enzyme is denatured why did you test 60°C?	Reaction temperature of 60℃ was tested since basically the lipase enzyme catalyst can be active at the temperature range of 20 ℃- 60℃ as reported by Samsumaharto (2008) and Thongprajukaew et al. (2010). This information has been added in the paragraph. However, in this work, the highest reaction conversion was obtain at 50℃. Thereaction conversion was decreasing at 50℃ and the higher temperature. This phenomenon was possibly occurred due to the enzyme denaturation.
13	Row 226: Delete the sentence "which conveyed that the highest lipase catalyst loading was 15%."	It has been deleted as suggested.
14	Conclusions: Expand on the conclusions. Given the process costs and the need for biodiesel purification, can the 41.46 % yield be considered advantageous? Can the process be applied on an industrial scale, or is optimization required to be effectively applicable?	The conclusion been expanded as suggested: “Based on the RSM analysis, it was revealed that reaction time and catalyst concentration had significant effects on the conversion of the interesterification reaction of CSO with ethyl acetate in the presence of lipase catalyst for biodiesel synthesis. The results of the analysis showed that the optimum conversion was 41.46% at the reaction temperature, reaction time, and catalyst concentration of 44.40°C, 5 hours, and 20%, respectively. The result was good, nonetheless, it is not economically feasible yet for industrial scale production and purpose with the current condition. Therefore, the further process intensification is necessary to enhance the reaction conversion and biodiesel yield.

REVIEWER 4

No	Reviewer Comment	Response
1	The Introduction could benefit from a scheme elucidating about the processes being discussed: the common transesterification and the interesterification proposed here.	The reaction schemes of transesterification and interesterification have been added in the introduction part (Equations 1 and 2).
2	In the Experimental Section a reference for the Design Expert software is missing.	The reference for Response Surface Methodology (RSM) with the Box Behnken Design (BBD) model on Design Expert software has been added in the introduction and method parts. The Levels of tested parameter for the BBD experiment are presented in Table 2.
3	The conversion in Figure 3 for the catalyst loading of 10% was ca. 14% at 40ºC and after 5h. Why is this result different from that reported in Figure 2, almost 2x, for the same conditions? And why have all the temperatures and reaction times been tested? At least I would expect the best condition from Figure 2 to be tested (40 ºC and 3h). Authors must address this.	Figure 3 has been revised and replaced with the revised version. All the temperatures and reaction times been tested to see the effects of reaction temperatures on the reaction conversion.
4	Again the data in Table 4 doesn't correlate with Figure 2, Please check and correct.	The data in Table 4 with catalyst concentration of 10% is also obtained from Figure 2 and actually the same.
5	Concerning data in Figure 7 showing the optimized reaction parameters. What I do not understand is that is how those parameters are worse than the data showing in Figure 3 for the reaction at 40 ºC after 3h and 10% catalyst, with conversion at almost 60%. Explanation must be provided.	The parameter in the design expert is already set between those temperature, reaction time and catalyst concentration. However, the data at 40 ℃ and 3 hour is not included in the BBD design. The Levels of tested parameter for the BBD experiment are presented in Table 2.

energies

Article

Synthesis of Biodiesel via Interesterification Reaction of *Calophyllum inophyllum* Seed Oil and Ethyl Acetate over Lipase Catalyst: Experimental and Surface Response Methodology Analysis

Ratna Dewi Kusumaningtyas [1,*], Normaliza Normaliza [1], Elva Dianis Novia Anisa [1], Haniif Prasetiawan [1], Dhoni Hartanto [1], Harumi Veny [2], Fazlena Hamzah [2], and Miradatul Najwa Muhd Rodhi [2]

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Abstract: Biodiesel has gained a rising consideration as an alternative to the fossil fuel as it is renewable, nontoxic, biodegradable, and feasible for mass production. Biodiesel can be produced from various types of vegetable oils. *Calophyllum inophyllum* seed oil (CSO) is among the prospective non edible vegetable oil as raw material for biodiesel synthesis. The most common process of the biodiesel manufacturing is the transesterification of vegetable oils which results in glycerol as a by-product. Thus, product purification is necessary. In this work, an alternative route of biodiesel synthesis through interesterification reaction of vegetable oil and ethyl acetate was conducted. By replacing alcohol with ethyl acetate, triacetin is produced as side product rather than glycerol. Triacetin has ability as a fuel additive which can increase the octane number of the fuel. Therefore, triacetin separation from biodiesel product is needless. Interesterification reaction is catalyzed by an alkaline catalyst or by lipase enzyme. In this study, biodiesel synthesis was carried out using lipase enzyme since it is a green and sustainable catalyst. The interesterification reaction of CSO with ethyl acetate in the presence of lipase catalyst was conducted using the molar ratio of CSO and ethyl acetate of 1:3. The reaction time, lipase catalyst concentration, and reaction temperature were varied at 1,2,3,4 ,5 hours, 10%,15%, 20%, and 30°C, 40°C, 50°C, 60°C, respectively. The experimental results were also analyzed using Response Surface Methodology (RSM) with the Box Behnken Design (BBD) model on Design Expert software. Data processing using RSM revealed that the optimum conversion and selectivity were 41.46% and 23.31%, respectively, obtained at the temperature reaction of 44.43°C, reaction time of 5 hours, and lipase catalyst concentration of 20%.

Keywords: biodiesel; *Calophyllum inophyllum* seed oil; interesterification; enzymatic, Box Behnken Design

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Energies* **2022**, *15*, x. https://doi.org/10.3390/xxxxx

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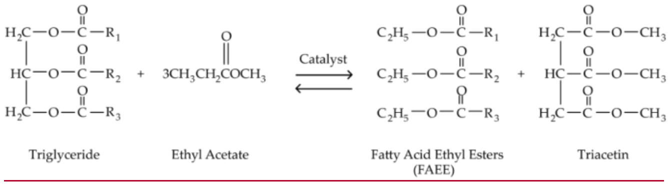
1. Introduction

Biodiesel is an alternative fuel for diesel engines which is synthesized from vegetable oils or animal fats. Biodiesel is one of the modern bioenergy which has several advantages. It has non-toxic properties, low emission rates and sulfur gas, just to name a few. Thus, biodiesel is a prospective alternative of the environmentally friendly diesel fuel [1]. Biodiesel is commonly derived from vegetable oil which has renewable characteristic. The main components of vegetable oils and animal fats are triglycerides, which can be converted into mono alkyl esters of long chain fatty acid or termed as fatty acid alkyl ester (biodiesel). Various types of vegetable oils can be applied as prospective raw materials for biodiesel preparation [2]. The widely used raw material for biodiesel synthesis in Indonesia is crude palm oil (CPO). CPO is abundantly available since it is produced on a large scale. However, the production of palm oil biodiesel competes with food needs. Therefore, other alternative vegetable oils are needed as raw materials for biodiesel. Non edible oil feedstocks are favorable to ensure the sustainability biodiesel production [3,4].

There are several non edible oils that have been widely investigated as biodiesel feedstocks such as silk–cotton or *Ceiba pentandra* seed oil [5], jatropha oil [4,6], castor oil [7], rubber seed oil [8], karanja oil [9], mahua oil, neem oil [10], waste cooking oil [11], palm fatty acid distillate [12] and *Calophyllum inophyllum* seed oil [13]. One among the potential non edible vegetable oils in Indonesia is *Calopyllum inophyllum* seed oil (CSO). It is also known as tamanu or nyamplung seed oil. Akram et al. [1] reported that *Calopyllum inophyllum* (tamanu) seed has oil content of 65-75%, which is higher than other nonedible seed oil plants such as jatropha seed oil (27-40%), rubber seed kernel oil (40-50%), and castor oil (45-65%). Rasyid et al. [14] reported that fatty acids composition of CSO comprises of oleic acid, palmitic acid, linoleic acid, and stearic acid. According to Adenuga et al. [13], CSO biodiesel meets the Australian, ASTM and EN standards. Therefore, CSO is suitable to be used as feed stock in the production of biodiesel. However, CSO contains high gummy substances, especially in the form of lipoid A (the hydratable phosphatides) and lipoid B (the non-hydratable phosphatides [15,16], waxes, and other impurities [17]. The existence of gum is unfavorable since it may lower the reaction rate which in consequence reduces the biodiesel yield. At industrial level, this condition leads to the economic disadvantage. Besides, the presence of phosphorus compounds can generate the carbon particle deposition in diesel engine which makes plugging in the engine filter, line, and injectors as well as reducing the engine performance [17,18]. Thus, degumming process is essential as the pretreatment step of the biodiesel feedstocks with high phosphorus content [19].~~However, CSO contains high gum, thus degumming process is necessary as the pretreatment step of the biodiesel feedstocks [15].~~

The most general method for making biodiesel is an alkaline catalyzed transesterification reaction by reacting vegetable oils with short chain alcohols such as methanol or ethanol [20,21]. However, transesterification reaction has a limitation in term of the potential for a saponification side reaction if the oil feedstock contains high free fatty acid (FFA) 2 mgKOH/g as described by Kusumaningtyas et al. [4] and Sebastian et al. [22]. Besides, transesterification reaction results in glycerol as by product which needs a separation process to obtain high purity biodiesel. Biodiesel purification from glycerol byproducts requires a series of separation steps which brings in high operation cost. To overcome these problems, it is necessary to apply the process of producing biodiesel which is glycerol-free through an interesterification reaction [23]. In the interesterification reaction, the use of methanol or ethanol is replaced with the methyl acetate or ethyl acetate. Interesterification of triglyceride yields triacetin co-product instead of glycerol [24]. Triacetin has advantage as a fuel additive, a good anti knocking and can enhance the octane number. The addition of triacetin to the fuel will also offer an environmental benefit since it reduces the exhaust smoke and NO emissions to some extent. It can be stated that the existence of triacetin co-product in biodiesel will contribute to the engine performance improvement [25]. It occurs since the addition of triacetin increases the oxygen content of the fuel [26]. Thus, it is unnecessary to separate triacetin from biodiesel products. Comparison between transesterification and interesterification reaction schemes are presented in Equations 1 and 2, respectively.



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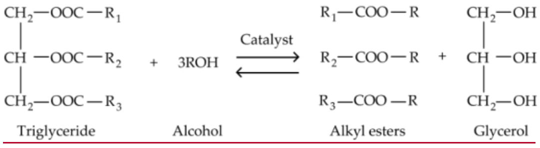

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To date, development of green energy through environmentally friendly processes becomes priority. One alternative towards green process is by reducing the use of chemical catalysts and switching to enzymatic catalyst (biocatalysts). The enzyme catalyzed biodiesel synthesis shows superiority compared to the chemically catalyzed one, mainly in terms of the lower energy requirement and natural conservation aspect. Lipase is the most common enzyme employed for biodiesel synthesis. There are two principal classes of lipase, free enzyme and immobilized enzyme. Free lipase enzyme is cheaper and simpler, thus it is feasible for large scale application [27]. Besides that, the use of lipase enzymes is very promising to overcome the disadvantages of alkaline catalysts related to occurrence of the undesired saponification reaction when the high FFA oil used as raw material. Lipase enzyme catalyst can be applied for the high FFA feedstock without any necessity of FFA removal as the pretreatment step. However, lipase biocatalysts are easily deactivated in an alcoholic environment since short-chain alcohols often cause irreversible loss of enzyme activity [28]. Therefore, the non-alcoholic interesterification route is preferable in order to maintain high biocatalyst activity and stability during the reaction. In the interesterification reaction, alcohol can be replaced by methyl acetate or ethyl acetate which is harmless for the lipase catalyst [29].

In this work, biodiesel was synthesized through lipase catalyzed interesterification of *Calopyllum inophyllum* seed oil (CSO) with ethyl acetate. The lipase enzyme used was the liquid free lipase from Novozyme. The effects of main parameter on the reaction conversion were studied experimentally. Process optimization was also conducted using Response Surface Methodology. Response Surface Methodology (RSM) is a collection of mathematical and statistical calculation techniques based on the compatibility of the empirical model with the experimental data obtained. RSM is useful for modeling and analysis of the problems that influences the response variables with the purpose of optimizing the response. Response Surface Methodology (RSM) analysis is useful in determining the operating conditions to achieve the desired conversion [30]. There are several experimental design models used in RSM, such as Box-Behnken Design (BBD), Control Composite Design (CCD) and Doehlert Design. The BBD has been broadly applied for biodiesel production optimization since it is effective, requires the smallest number of trials than the other designs [31]. In this study, optimization of the interesterification reaction of CSO in the presence of lipase enzyme catalyst was performed using BBD in RSM analysis to determine the best reaction operation condition which resulted in the highest reaction conversion.

2. Materials and Methods

2.1. Materials

The materials used were: *Calophyllum inophyllum* seed oil (CSO) from UMKM Samtamanu Cilacap, Indonesia, ethyl acetate p.a (Merck), liquid lipase catalyst (Eversa Transform 2.0 from Novozyme), phosphoric acid p.a (Merck), KOH p.a (Merck), oxalic acid p.a (Merck), ethanol p.a (Merck), and aquadest from Indrasari chemicals store, Semarang, Indonesia. The catalyst used was liquid (free form) lipase Eversa Transform 2.0 (ET 2.0) from Novozyme, which is made from genetically modifed Aspergillus oryzae microorganism [32]. Commonly, lipase are active in broad range of temperature from 20-60 °C [33,34] and the ET 2.0 was reported to work satisfactorily up to the reaction temperature of 60°C [35].

2.2. Method

2.2.1. Degumming

Crude CSO as much as 100 mL was put into a 250 mL flat bottom flask. The oil was then heated using hot plate equipped with a magnetic stirrer at a speed of 600 rpm until the temperature reached 70°C. Phosphoric acid was subsequently added to the hot oil with the amount of 0.3% (w/w) of CSO and heated for 25 minutes. The oil was cooled at room temperature and put into a 500 mL separatory funnel. Distilled water at a temperature of 40°C was thereafter added with a volume of 5% of the CSO volume. Subsequent to the degumming process, the mixture was settled in the separatory funnel for 24 hours to attain the completion of the separation between the CSO and the residue. After the 24 hours decantation process, the two layers were appeared. The top layer of brownish yellow color was the refined CSO, while the dark brown bottom layer was gum, impurities, and water which should be separated. Let the mixture in the separatory funnel for to attain the completion of the separation between the oil and the residue. The degummed CSO was then separated from the residue and was heated in the oven with a temperature of 105°C to reduce the water content [30]. CSO was then analyzed to determine its physical properties (density, viscosity, and acid number).

2.2.2. Interesterification Reaction

After the degumming process, CSO was used as the raw material for biodiesel synthesis via interesterification reaction. CSO as much as 100 mL was introduced into a 250 mL flat bottom three neck flask and added with 10% (w/w) liquid lipase. The mixture was heated using a hot plate equipped with a magnetic stirrer with the stirring speed of 600 rpm until it reached the desired temperature. On the other hand, ethyl acetate was warmed up separately up to the similar temperature. When the reactants attained the specified temperature, ethyl acetate was subsequently poured into the reactor and mixed with the CSO and lipase. This incident was recorded as the reaction time of zero (t = 0 h). The ratio of CSO to ethyl acetate was fixed at 1:3. The stoichiometric ratio of the reactants was employed to investigate the optimum reaction conversion that can be obtained without any excess reactant (ethyl acetate) as it was conducted by Manurung et al. [36]. The reaction temperature was varied at 30°C, 40 °C, 50°C and 60°C.The reaction temperature was varied at 30°C, 40 °C, 50°C and 60°C. After the specified temperature was reached, the ethyl acetate with the similar temperature was added with the ratio of CSO to ethyl acetate of 1:3 (stoichiometric ratio). The reaction time was 1-5 h. Sample was taken periodically every 1 hour of the reaction time. Prior to the analysis, sample was centrifuged with the centrifugation speed of 40,000 rpm for 15 minutes to separate between biodiesel product and the catalyst residue. The sample analysis was carried out using Gas Chromatography-Mass Spectroscopy (GC-MS). The independent variables for the experimental work is shown in Table 1.

Table 1. Independent Variables.

Variable	Values
Reaction Time	1,2,3,4, and 5 hours
Reaction Temperature	30°C, 40°C, 50°C, and 60°C
Catalyst Concentration	10%, 15%, and 20%

2.2.3. Gas Chromatography-Mass Spectroscopy (GC-MS)

Analysis of the fatty acid composition of the biodiesel was determined using a Gas Chromatography-Mass Spectrometer (GC-MS QP2010 SE) with a column flow rate of 1.20 mL/min, an oven column temperature of 65°C, a pressure of 74.5 kPa, and an injecttion temperature of 250°C with split injection mode. The internal standard used was heptadecanoic acid, methyl ester.

2.2.4. Response Surface Methodology

The GC-MS data was utilized to determine the reaction conversion. The result was subsequently analyzed using Response Surface Methodology (RSM) with the Box Behnken Design (BBD) model on Design Expert software to determine the optimum operation condition. The BBD is a vigorous and extensively applied model for biodiesel synthesis optimization [31]. The Levels of tested parameter for the BBD experiment are presented in Table 2.

Table 2. Levels of Tested Parameter for Box Behnken Design (BBD).

Independent Factor	Units	Symbol	Level	
			Low	High
Temperature	°C	A	30	50
Reaction Time	hours	B	1	5
Catalyst Concentration	%	C	10	20

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3. Results and Discussion

3.1. Effects of the Degumming Process on the CSO Properties

Naturally, the crude CSO contains phospholipids, sterols, free fatty acids, waxes, oil-soluble pigments and hydrocarbons. Degumming is the initial and essential refining process of crude vegetable oil to remove phospholipids and gums [37]. To date, there are various methods that can be employed to degum vegetable oil, explicitly water, acid, enzymatic, and membrane degumming techniques. Water degumming and acid degumming is the most used methods for vegetable degumming at industrial scale [38]. Those methods are simple, easy, and low cost. However, water degumming is less effective compared to the acid method. Gum comprises of the hydratable (HP) and non-hydratable phosphatides (NHP). HP can be eliminated using water. In contrast, NHP cannot be eradicated via water degumming [15,16]. Thus, acid degumming method is more advantageous in which it can remove both the HP and NHP. However, water degumming is less effective compared to the acid degumming. Thus, acid degumming method is more advantageous. The most used acid for degumming process are phosphoric or citric acid with the suggested concentration between 0.05 – 2% w/w oil [39]. In this work, 0.3% phosphoric acid was applied [23,40]. After the degumming process, the black color of the CSO turns into reddish yellow due to the loss of the phospholipids compound in crude CSO. Phospholipids can form dark color in vegetable oil as a result of the autoxidation process of these compounds during storage. Condensation reaction between the amino groups in phospholipids with aldehydes will yield melanophosphatides compound which give dark color to the oil [41]. Thus, removal of phospholipids in CSO will reduce the occurrence of the above mention reaction and diminish the color intensity.

Degumming process can improve the properties of CSO. In this work, the density, viscosity, and acid number of crude and degummed CSO were tested experimentally. The effects of the degumming process on the physicochemical properties of CSO is indicated in Table 3. It was obvious that the density and viscosity of the CSO were slightly decreasing after the degumming process due to the removal of gum and other impurities. The effect of degumming process on the CSO properties is indicated in Table 2. It was revealed that the density and viscosity of the CSO were slightly decreasing after the degumming process. It occurred to the removal of gum and other impurities. Besides, degumming also lead to the lessening of the acid number of the oil which was attributable to the decrease of the acid number and the free fatty acid (FFA) existence in the oil during the degumming process. This phenomenon is in line with the data reported by Adekunle et al.[42]. Fatty acid composition of the CSO after undergoing degumming process was presented in the Table 3, based on the interpretation of the chromatogram shown in Figure 1.

Table ~~2~~3. Effects of Degumming Process on the Properties of CSO.

CSO Properties	Before Degumming	After Degumming
Density (kg/m^3)	941	937
Viscosity (mm^2/s)	63.42	59.73
Acid Number (mg KOH/~~kg~~ CSO)	64.62	48.24
Free Fatty Acid Content (%)	32.47	24.25

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Table ~~3~~4. Fatty Acid Composition of the CSO after Degumming Process.

Fatty Acid	Molecular Weight (g/mol)	Area (%)
Palmitic Acid	256.22	7.82
Linoleic Acid	280.45	16.82
Oleic Acid	282.52	26.62
Stearic Acid	284.47	8.86
Arachidic Acid	312.54	0.31

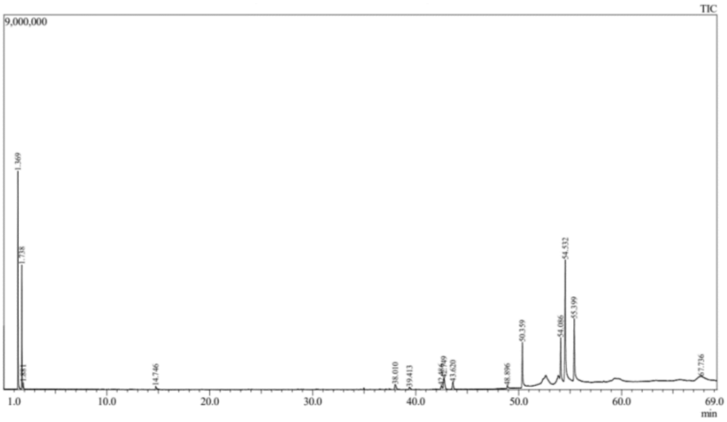


Figure 1. Chromatogram of the CSO after Degumming Process.

3.2. Effects of the Temperature on the Interesterification Reaction Conversion

After the degumming process, CSO directly underwent interesterification process with ethyl acetate in the presence of lipase catalyst. ~~A specific pre-treatment for reducing the FFA content of CSO was not considered necessary since the FFA presentation in the oil does not affect the enzymatic catalyzed reaction. The influences of the reaction time and temperature on the reaction conversion were observed at the fixed lipase catalyst concentration of 10% (w/w). The result is exhibited in Figure 2.~~ As depicted in Table 2, the crude and degummed CSO contained high FFA of 32.47% and 24.25%, respectively, which were over 2 mgKOH/g. However, enzymatic reaction is generally insensitive to the existence of the FFA impurities in the oil feedstock. The possibility of the undesired saponification reaction can be neglected. Therefore, a specific pre-treatment for reducing the FFA content of CSO is considered unnecessary since the FFA presentation in the oil does not affect the enzymatic catalyzed reaction [22,43].

The influences of the reaction time and temperature on the reaction conversion were observed at the fixed lipase catalyst concentration of 10% (w/w). The result is exhibited in Figure 2. Basically, lipase are active in the temperature range of 20-60 °C [33,34] and the ET 2.0 lipase can work up to 60°C [35]. In this work,~~Based on the experimental data,~~ it was disclosed that the highest reaction conversion was 54.99%, obtained at the reaction temperature of 40°C and the reaction time of 3 hours. Commonly, the reaction conversion is enhanced with the increasing of the reaction temperature and reaction time since at the higher temperature, the viscosity of the liquid decreases, causing the higher solubility of reactants, mass diffusion, rate of reaction, and conversion [44]. However, in this work, the reaction conversion decreased when the reaction was performed at 50°C for 5 hours. This trend was in accordance with the result recorded by Gusniah et al. [45]. It was reported that the lipase enzyme catalyst activity in the transesterification reaction of waste cooking oil reached the optimum performance at 40 °C and it declined to some extent when the temperature rose to 50 °C. It happened since the lipase catalyst began to denature, causing the damage of the active part of the enzyme at the higher temperature. Beyond the optimum temperature, lipase catalyst becomes unstable and misplaces its tertiary structure which causes the shortfall of its activity [44]. It thus leads to the declining of the reaction rate and conversion. Generally, the lipase enzyme reached the highest activity at the temperature of 40°C – 45°C as stated by Murtius et al. [46], Ayinla et al. [47], and Yazid [48].

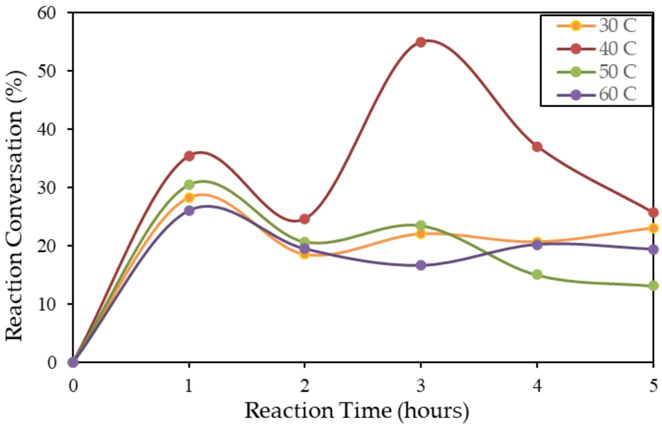


Figure 2. Effects of the reaction time and temperature on the reaction conversion at fixed lipase catalyst concentration of 10% (w/w).

3.3. Effects of the Catalyst Concentration e on the Interesterification Reaction Conversion

Investigation on the influence of the lipase catalyst concentration was carried out at the molar ratio of oil to ethyl acetate of 1:3, reaction temperature of 40°C and reaction time of 5 hours. The lipase concentration studied was 10%, 15%, and 20%. Figure 3 has shown that the optimum catalyst concentration was 15%, resulting in the reaction conversion of 28.445%. This result was in agreement with the resume conveyed by Gusniah et al. [45] which conveyed that the highest lipase catalyst loading was 15%. However, the reaction conversion in this work was still low compared to the other research. It came about since the reaction was conducted with the low molar ratio of oil and ethyl acetate (1:3) and short reaction time (5 h). As comparison, Sun et al. [49] achieved the 94.2% yield for the transesterification of semen Abutili seed oil in the presence of a similar lipase catalyst but at the far excess molar ratio of oil and alcohol (1:7) and longer reaction time (11 h).

Subhedar and Gogate [29] carried out the interesterification reaction of waste cooking oil and methyl acetate using immobilized lipase obtained from *Thermomyces lanuginosus* (Lipozyme TLIM) as a catalyst. The optimum were demonstrated at the oil : methyl acetate molar ratio of 1:12, enzyme concentration of 6% (w/v), reaction temperature of 40 °C and reaction time of 24 h. Biodiesel yield was 90.1% under these operation conditions, which was higher than this work. The higher reaction conversion can be provided since the reaction was conducted on an excessive molar ratio and extensively longer reaction time (24 h). Besides, this reaction employed an immobilized lipase, in which the lipase was attached on the carrier substrate, which led to the higher product purity. Thus, it can be deduced that the lipase catalyzed reaction this work resulted in a lower conversion due to a shorter reaction time and low molar ratio of the reactants, which was not excess than the stoichiometric ratio. Besides that, utilization of the liquid lipase can reduce the apparent concentration of biodiesel yield since it can act as an impurity in the final biodiesel product.

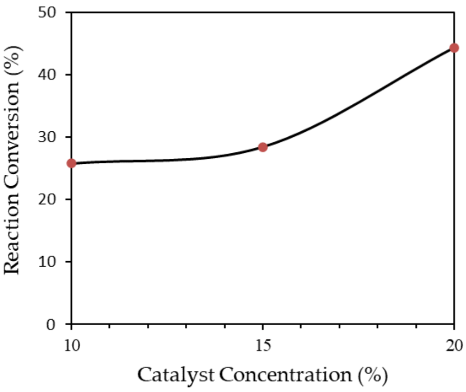


Figure 3. Effects of the Lipase Catalyst Concentration at the Reaction Temperature of 40°C and Reaction Time of 5 Hours.

3.4. Response Surface Methodology (RSM) Analysis

Response surface methodology (RSM) is the broadest employed method of experimental design for optimization. This technique applies mathematical and statistical method for modeling analyzing a process which involves numerous. The purpose of the RSM is to optimize the response variables [29]. The factors that influence the process are entitled dependent variables. Meanwhile, the responses are named as dependent variables. Therefore, analysis using Response Surface Methodology (RSM) is useful in designing operating conditions to achieve the targeted conversion. Many literatures have shown that the most applicable tools in RSM for optimization of biodiesel production process are the Box Behnken Design (BBD) and Central Composite Design (CCD) tools [50]. BBD is favored than CCD since it provides higher efficiency but more economical [51,52]. To evade the failure and extreme reaction variables value, BBD is limited to three levels. Hence, BBD typically has no factorial points. The BBD tool was also utilized by Sharma et al. [53] and Rokni et al. [54].

In this study, BBD was applied to obtain the optimal operating conditions for biodiesel synthesis via interesterification reaction. The Box Behnken Design (BBD) tool in RSM was used to evaluate the effect of the independent variables (reaction time, reaction temperature, and catalyst concentration). The experimental data of the reaction conversion compared with the prediction using BBD is exhibited in Table ~~4~~5.

The parameters used to obtain the appropriate model to predict the response results must match the observed model to the experimental data. Analysis of variance (ANOVA) was accomplished using the most common types of polynomial models, namely linear, interactive (2FI), quadratic and cubic can be applied to predict the response variables of experimental data. Several parameters such as sequential p-value, lack of fit p-value, adjusted R¬2, predicted R2 and Adiq precision were used to conclude the most suitable type of model for the optimization of the conversion of biodiesel produced from CSO. In this study, optimization using RSM was initially performed using the quadratic model. The ANOVA quadratic model is presented in Table ~~5~~6.

Table ~~4~~5. Experimental Data of the Reaction Conversion and the Prediction Using BBD.

Run	Temperature, ℃	Reaction Time, hr	Catalyst Concentration, % w/w	Reaction Conversion, %		Error, %
				Experiment	**Prediction**	
1	40	3	15	12.92	18.15	40.4
2	30	3	10	22.08	19.877	9.97
3	40	5	20	48.92	48.03	1.8
4	30	3	20	27.75	27.33	1,5
5	40	5	10	25.77	26.66	3.4
6	40	3	15	18.27	18.15	0.6
7	30	1	15	23.41	24.72	5.6
8	50	5	15	14.90	13.59	8.8
9	40	1	20	13.55	12,66	6.5
10	30	5	15	22.10	23.41	5.9
11	40	3	15	23.25	18.15	21.9
12	40	1	10	35.43	36.32	2.5
13	50	1	15	26.82	25.51	4.8
14	50	3	20	12.01	14.22	18.3
15	50	3	10	23.53	23.96	1.7

Table ~~5~~6. ANOVA of the Quadratic Model.

Source	Sum of Squares	DF	Mean Square	F-value	p-value	
Model	1007.96	9	112.00	2.13	0.2098	not significant
A-Temperature	40.83	1	40.83	0.7760	0.4187	
B-Reaction Time	19.45	1	19.45	0.3696	0.5698	
C-Catalyst Concentration	2.61	1	2.61	0.0497	0.8325	
AB	28.14	1	28.14	0.5348	0.4974	
AC	73.90	1	73.90	1.40	0.2892	
BC	507.01	1	507.01	9.64	0.0267	
A^2	32.27	1	32.27	0.6134	0.4690	
B^2	161.73	1	161.73	3.07	0.1399	
C^2	139.90	1	139.90	2.66	0.1639	
Residual	263.06	5	52.61			
Lack of Fit	209.74	3	69.91	2.62	0.2881	not significant
Pure Error	53.32	2	26.66			
Cor Total	1271.02	14				

Based on the quadratic model, it was revealed that the p-value was 0.~~2998~~2098, which did not meet the requirement of the p-value < 0.05 as demonstrated in Tabel ~~5~~6. Hence, the quadratic model was not significant for this case. To overcome this obstacle, modified model was developed to obtain the significant model [55]. Modification of the model shown that the reduced cubic model made evident of the significant model (Table ~~6~~7).

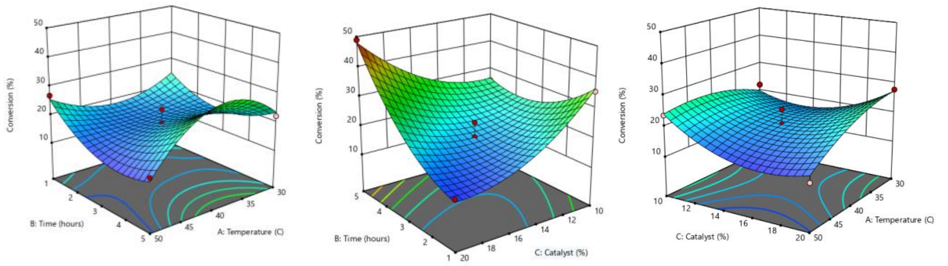
Table 6. ANOVA of the Reduced Cubic Model.

Source	Sum of Squares	DF	Mean Square	F-value	p-value	
Model	1197.58	10	119.76	6.52	0.0428	significant
A-Temperature	40.83	1	40.83	2.22	0.2102	
B-Reaction Time	165.26	1	165.26	9.00	0.0399	
C-Catalyst Concentration	2.61	1	2.61	0.1423	0.7252	
AB	28.14	1	28.14	1.53	0.2834	
AC	73.90	1	73.90	4.03	0.1153	
BC	507.01	1	507.01	27.62	0.0063	
A^2	32.27	1	32.27	1.76	0.2555	
B^2	161.73	1	161.73	8.81	0.0412	
C^2	139.90	1	139.90	7.62	0.0508	
A^2B	189.62	1	189.62	10.33	0.0325	
Residual	73.44	4	18.36			
Lack of Fit	20.12	2	10.06	0.3773	0.7260	not significant
Pure Error	53.32	2	26.66			
Cor Total	1271.02	14				
Adeq Precision	9.6401					

It was found that the p-value of the modified cubic model was 0.0428, which has met the requirement of p-value > 0.05. Furthermore, the value of lack-of-fit was examined. Lack-of-fit is a test that analyzes how satisfactory the full models suit with the data. Model with a significant lack-of-fit should not be applied for the prediction. In this work, the lack-of-fit was not significant, which means that the model can be employed for the prediction. The value of adeq precision is the measure of the ratio of the signal to the disturbance. The expected value of the ratio is > 4 [50]. This model provided the value of the Adeq precision of 9.6401, indicating that the model is proper. Therefore, the reduced cubic model can be used to describe the response on the reaction conversion. The empirical correlation of the reduced cubic model in the form of polynomial orde for the reaction conversion in the CSO esterification is displayed in Equation ~~1~~3.

$$\text{Conversion (\%)} = 18.15 - 2.26\,A + 6.43\,B - 0{,}5715\,C - 2.65\,AB - 4.30\,AC + 11.26\,BC - 2.96\,A^2 + 6.62\,B^2 + 6.16\,C^2 - 9.74\,A^2B \qquad (1)$$

Where, A is the reaction temperature (°C), B is the reaction time (min) and C is the Catalyst Concentration (%). Based on the reduced cubic model, analysis on the effects and the process variables interaction on the response variable were carried out using 3D RSM graphs. The results are presented in Figure 4.



(a) (b) (c)

Figure 4. The 3D RSM graph representing the effects of reaction time, temperature and catalyst concentration on the reaction conversion.

Figure 4(a) shows the interaction between temperature and reaction time which gives a significant change to the increase in conversion. It was found that there was an increase in conversion at the temperature of 40°C and reaction time of 5 hours. This phenomenon is in line with the investigation of Handayani et al. [55], which reported that the optimum conversion was obtained at the temperature of 45°C and reaction time of 6 hours. Figure 4(b) discloses that the longer the reaction time employed, the higher the conversion achieved. It happened since the reaction time is directly proportional to the amount of product yielded. Li et al. [56] conveyed that the reaction conversion of *Pseudomonas cepacia* lipase was low at the reaction time of 3 h. (44%). On the contrary, it rose to 73% when the reaction time extended to 12 h. Figure 4(c) bares that the lower the reaction temperature applied, the higher conversion resulted in. It occurred since the lipase enzyme activity works at the temperature of 30°C – 45°C. In the enzymatic reaction, the increase of the temperature will promote the reaction rate and accordingly the reaction conversion due to the decrease of the viscosity which enhance the solubility of oil and methanol. However, at a certain temperature, reaction conversion declines evoking the enzyme deactivation [57]. Meanwhile, the addition of the catalyst concentration increased the reaction conversion. This result was in line with the description of Gusniah et al. [45]. It was justified that the increasing amount of the enzyme employed will provide a higher amount of the available active sites for the reaction, leading to the higher reaction conversion.

The Derringer method is utilized to optimize the response which is characterized by the presence of a desirability function. Desirability shows how close the optimization results are to the optimum point. The desirability function (DF) is constructed on the transformation of all the acquired responses from different scales into a scale-free value. The values of DF are ranging from 0 to 1. The value 0 means that the factors provide an undesirable response. On the other hand, the value 1 denotes the optimal performance of the evaluated factor [58]. Hence, the desirability value which is close to 1 is the expected value. However, the RSM analysis is not only to find the desirability value which is equal to 1, but to determine the conditions that match expectations. The multiple correlation coefficient (R^2) was closed to 1. It denoted that the models have good predictive ability. Based on the optimization results, the optimum conversion was 41.46%, attained at a temperature of 44.43°C, a reaction time of 5 hours and a catalyst concentration of 20%, with a desirability value of 0.733.

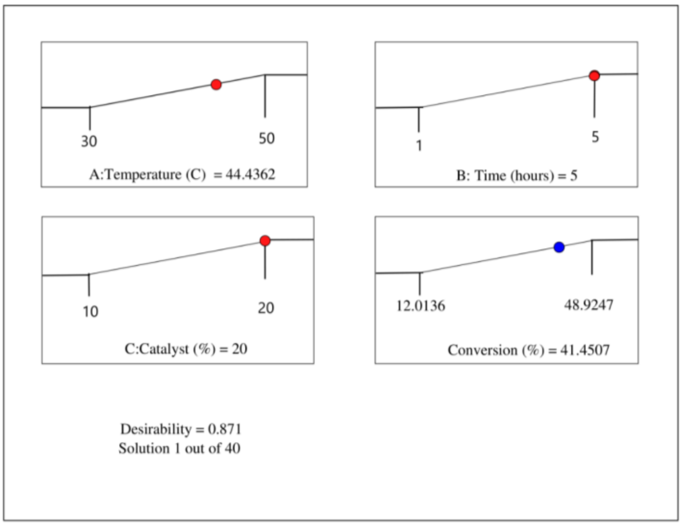


Figure 7. Reaction Conversion Optimization Using RSM

5. Conclusions

Based on the RSM analysis, it was revealed that reaction time and catalyst concentration had significant effects on the conversion of the interesterification reaction of CSO with ethyl acetate in the presence of lipase catalyst for biodiesel synthesis. The results of the analysis showed that the optimum conversion was 41.46% at the reaction temperature, reaction time, and catalyst concentration of 44.40°C, 5 hours, and 20%, respectively. The result was good, nonetheless, it is not economically feasible yet for industrial scale production and purpose with the current condition. Therefore, the further process intensification is necessary to enhance the reaction conversion and biodiesel yield.

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Journal	Energies (https://www.mdpi.com/journal/energies) (ISSN 1996-1073)
Manuscript ID	energies-1944330
Type	Article
Title	Synthesis of Biodiesel via Interesterification Reaction of Calophyllum inophyllum Seed Oil and Ethyl Acetate over Lipase Catalyst: Experimental and Surface Response Methodology Analysis
Authors	Ratna Dewi Kusumaningtyas * , Normaliza Normaliza , Elva Dianis Novia Anisa , Haniif Prasetiawan , Dhoni Hartanto , Harumi Veny , Fazlena Hamzah , Miradatul Najwa Muhd Rodhi
Topic	Chemical and Biochemical Processes for Energy Sources (https://www.mdpi.com/topics/chemical)
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Does the introduction provide sufficient background and include all relevant references?	(x)	( )	( )	( )
Are all the cited references relevant to the research?	(x)	( )	( )	( )
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Are the results clearly presented?	( )	(x)	( )	( )

Are the conclusions supported by the results?	( )	(x)	( )	( )

Comments and Suggestions for Authors	I believe the authors can not mention that they achieved optimization owing low conversion values obtained. In fact in its response they said "We believe that this work will give valuable contribution for the future work in the area of green technology for biodiesel production". Please change the optimization focus.
Submission Date	15 September 2022
Date of this review	05 Oct 2022 15:28:50

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Are the results clearly presented?	(x)	( )	( )	( )

Are the conclusions supported by the results?	(x)	( )	( )	( )

Comments and Suggestions for Authors	The requested corrections were extensively carried out by the authors. Furthermore, I would like to take this opportunity to congratulate the authors, I find this study extremely relevant and interesting.
Submission Date	15 September 2022
Date of this review	07 Oct 2022 15:17:30

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Comments and Suggestions for Authors	Authors have provided a revised manuscript addressing all issues raised in the original manuscript. In my view the manuscript is suitable for publicaiton.
Submission Date	15 September 2022
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RESPONSE TO REVIEWER

REVIEWER 2

No	Reviewer Comment	Response
1	I believe the authors cannot mention that they achieved optimization owing low conversion values obtained. In fact in its response they said "We believe that this work will give valuable contribution for the future work in the area of green technology for biodiesel production".	Thank you for your valuable review. Based on your suggestion, we change the terminology of "optimal conversion" into "the highest conversion" or "the local optimum achieved within the range of parameters values". We put the revised sentences in the following sections: **1. Abstract** "Data processing using RSM revealed that the highest conversion within the studied parameter range was 41.46%, obtained at the temperature reaction of 44.43°C, reaction time of 5 hours, and lipase catalyst concentration of 20%". **2. Introduction** "In this investigation, analysis using BBD in RSM was performed to determine the best reaction operation condition which resulted in the highest reaction conversion of the interesterification reaction of CSO in the presence of lipase enzyme catalyst within the ranges of the parameter values studied". **3. Result and Discussion** *3.4. Response Surface Methodology (RSM) Analysis* **a**. "In this study, BBD was applied to obtain the best operating conditions and highest conversion for biodiesel synthesis via interesterification reaction within the ranges of the parameter values studied." **b.** "Based on the RSM analysis results, the highest reaction conversion was 41.46%, attained at a temperature of 44.43°C, a reaction time of 5 hours and a catalyst concentration of 20%, with a desirability value of 0.733. This

		finding can be stated as the local optimum within the certain parameter range studied, which is worthwhile as a basis for developing process intensification in order to obtain a higher conversion”. **4. Conclusion** “The results of the analysis showed that the highest reaction conversion was 41.46% at the reaction temperature, reaction time, and catalyst concentration of 44.40°C, 5 hours, and 20%, respectively, which can be stated as the local optimum.”

REVIEWER 3

No	Reviewer Comment	Response
1	The requested corrections were extensively carried out by the authors. Furthermore, I would like to take this opportunity to congratulate the authors, I find this study extremely relevant and interesting	Thank you very much for your time to review our manuscript. Your essential suggestion and positive recommendation have significantly improved the quality of this paper. We greatly appreciate it.

REVIEWER 4

No	Reviewer Comment	Response
1	Authors have provided a revised manuscript addressing all issues raised in the original manuscript. In my view the manuscript is suitable for publication.	Thank you very much for your time to review our manuscript. Your precious suggestion and encouraging recommendation have significantly improved the quality of this paper. It is highly respected.

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Synthesis of Biodiesel via Interesterification Reaction of *Calophyllum inophyllum* Seed Oil and Ethyl Acetate over Lipase Catalyst: Experimental and Surface Response Methodology Analysis

Ratna Dewi Kusumaningtyas [1,*], Normaliza Normaliza [1], Elva Dianis Novia Anisa [1], Haniif Prasetiawan [1], Dhoni Hartanto [1], Harumi Veny [2], Fazlena Hamzah [2], and Miradatul Najwa Muhd Rodhi [2]

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Abstract: Biodiesel has gained a rising consideration as an alternative to the fossil fuel as it is renewable, nontoxic, biodegradable, and feasible for mass production. Biodiesel can be produced from various types of vegetable oils. *Calophyllum inophyllum* seed oil (CSO) is among the prospective non edible vegetable oil as raw material for biodiesel synthesis. The most common process of the biodiesel manufacturing is the transesterification of vegetable oils which results in glycerol as a by-product. Thus, product purification is necessary. In this work, an alternative route of biodiesel synthesis through interesterification reaction of vegetable oil and ethyl acetate was conducted. By replacing alcohol with ethyl acetate, triacetin is produced as side product rather than glycerol. Triacetin has ability as a fuel additive which can increase the octane number of the fuel. Therefore, triacetin separation from biodiesel product is needless. Interesterification reaction is catalyzed by an alkaline catalyst or by lipase enzyme. In this study, biodiesel synthesis was carried out using lipase enzyme since it is a green and sustainable catalyst. The interesterification reaction of CSO with ethyl acetate in the presence of lipase catalyst was conducted using the molar ratio of CSO and ethyl acetate of 1:3. The reaction time, lipase catalyst concentration, and reaction temperature were varied at 1,2,3,4 ,5 hours, 10%,15%, 20%, and 30°C, 40°C, 50°C, 60°C, respectively. The experimental results were also analyzed using Response Surface Methodology (RSM) with the Box Behnken Design (BBD) model on Design Expert software. Data processing using RSM revealed that the highest ~~optimum~~ conversion ~~and selectivity were~~ within the studied parameter range was 41.46%~~ and 23.31%, respectively~~, obtained at the temperature reaction of 44.43°C, reaction time of 5 hours, and lipase catalyst concentration of 20%.

Keywords: biodiesel; *Calophyllum inophyllum* seed oil; interesterification; enzymatic, Box Behnken Design

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Energies* **2022**, *15*, x. https://doi.org/10.3390/xxxxx

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1. Introduction

Biodiesel is an alternative fuel for diesel engines which is synthesized from vegetable oils or animal fats. Biodiesel is one of the modern bioenergy which has several advantages. It has non-toxic properties, low emission rates and sulfur gas, just to name a few. Thus, biodiesel is a prospective alternative of the environmentally friendly diesel fuel [1]. Biodiesel is commonly derived from vegetable oil which has renewable characteristic. The main components of vegetable oils and animal fats are triglycerides, which can be converted into mono alkyl esters of long chain fatty acid or termed as fatty acid alkyl ester (biodiesel). Various types of vegetable oils can be applied as prospective raw materials for biodiesel preparation [2]. The widely used raw material for biodiesel synthesis in Indonesia is crude palm oil (CPO). CPO is abundantly available since it is produced on a large scale. However, the production of palm oil biodiesel competes with food needs. Therefore, other alternative vegetable oils are needed as raw materials for biodiesel. Non edible oil feedstocks are favorable to ensure the sustainability biodiesel production [3,4].

There are several non edible oils that have been widely investigated as biodiesel feedstocks such as silk–cotton or *Ceiba pentandra* seed oil [5], jatropha oil [4,6], castor oil [7], rubber seed oil [8], karanja oil [9], mahua oil, neem oil [10], waste cooking oil [11], palm fatty acid distillate [12] and *Calophyllum inophyllum* seed oil [13]. One among the potential non edible vegetable oils in Indonesia is *Calopyllum inophyllum* seed oil (CSO). It is also known as tamanu or nyamplung seed oil. Akram et al. [1] reported that *Calopyllum inophyllum* (tamanu) seed has oil content of 65-75%, which is higher than other nonedible seed oil plants such as jatropha seed oil (27-40%), rubber seed kernel oil (40-50%), and castor oil (45-65%). Rasyid et al. [14] reported that fatty acids composition of CSO comprises of oleic acid, palmitic acid, linoleic acid, and stearic acid. According to Adenuga et al. [13], CSO biodiesel meets the Australian, ASTM and EN standards. Therefore, CSO is suitable to be used as feed stock in the production of biodiesel. However, CSO contains high gummy substances, especially in the form of lipoid A (the hydratable phosphatides) and lipoid B (the non-hydratable phosphatides [15,16], waxes, and other impurities [17]. The existence of gum is unfavorable since it may lower the reaction rate which in consequence reduces the biodiesel yield. At industrial level, this condition leads to the economic disadvantage. Besides, the presence of phosphorus compounds can generate the carbon particle deposition in diesel engine which makes plugging in the engine filter, line, and injectors as well as reducing the engine performance [17,18]. Thus, degumming process is essential as the pretreatment step of the biodiesel feedstocks with high phosphorus content [19].~~However, CSO contains high gum, thus degumming process is necessary as the pretreatment step of the biodiesel feedstocks [15].~~

The most general method for making biodiesel is an alkaline catalyzed transesterification reaction by reacting vegetable oils with short chain alcohols such as methanol or ethanol [20,21]. However, transesterification reaction has a limitation in term of the potential for a saponification side reaction if the oil feedstock contains high free fatty acid (FFA) 2 mgKOH/g as described by Kusumaningtyas et al. [4] and Sebastian et al. [22]. Besides, transesterification reaction results in glycerol as by product which needs a separation process to obtain high purity biodiesel. Biodiesel purification from glycerol by-products requires a series of separation steps which brings in high operation cost. To overcome these problems, it is necessary to apply the process of producing biodiesel which is glycerol-free through an interesterification reaction [23]. In the interesterification reaction, the use of methanol or ethanol is replaced with the methyl acetate or ethyl acetate. Interesterification of triglyceride yields triacetin co-product instead of glycerol [24]. Triacetin has advantage as a fuel additive, a good anti knocking and can enhance the octane number. The addition of triacetin to the fuel will also offer an environmental benefit since it reduces the exhaust smoke and NO emissions to some extent. It can be stated that the existence of triacetin co-product in biodiesel will contribute to the engine performance improvement [25]. It occurs since the addition of triacetin increases the oxygen content of the fuel [26]. Thus, it is unnecessary to separate triacetin from biodiesel products. Comparison between transesterification and interesterification reaction schemes are presented in Equations 1 and 2, respectively.

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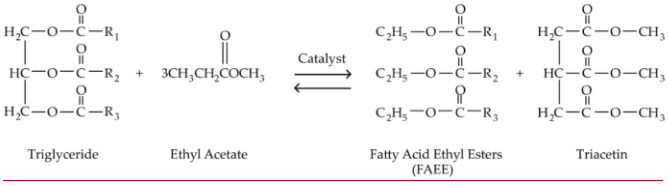

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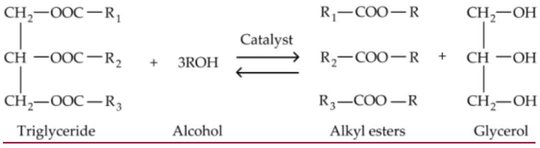

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To date, development of green energy through environmentally friendly processes becomes priority. One alternative towards green process is by reducing the use of chemical catalysts and switching to enzymatic catalyst (biocatalysts). The enzyme catalyzed biodiesel synthesis shows superiority compared to the chemically catalyzed one, mainly in terms of the lower energy requirement and natural conservation aspect. Lipase is the most common enzyme employed for biodiesel synthesis. There are two principal classes of lipase, free enzyme and immobilized enzyme. Free lipase enzyme is cheaper and simpler, thus it is feasible for large scale application [27]. Besides that, the use of lipase enzymes is very promising to overcome the disadvantages of alkaline catalysts related to occurrence of the undesired saponification reaction when the high FFA oil used as raw material. Lipase enzyme catalyst can be applied for the high FFA feedstock without any necessity of FFA removal as the pretreatment step. However, lipase biocatalysts are easily deactivated in an alcoholic environment since short-chain alcohols often cause irreversible loss of enzyme activity [28]. Therefore, the non-alcoholic interesterification route is preferable in order to maintain high biocatalyst activity and stability during the reaction. In the interesterification reaction, alcohol can be replaced by methyl acetate or ethyl acetate which is harmless for the lipase catalyst [29].

In this work, biodiesel was synthesized through lipase catalyzed interesterification of *Calopyllum inophyllum* seed oil (CSO) with ethyl acetate. The lipase enzyme used was the liquid free lipase from Novozyme. The effects of main parameter on the reaction conversion were studied experimentally. Analysis using Response Surface Methodology was also carried out to investigate the best reaction conversion obtained within the values range of the studied independent variables. Process optimization was also conducted using Response Surface Methodology. Response Surface Methodology (RSM) is a collection of mathematical and statistical calculation techniques based on the compatibility of the empirical model with the experimental data obtained. Generally, RSM is useful for modeling and analysis of the problems that influences the response variables with the purpose of optimizing the response. ~~Response Surface Methodology (~~RSM~~)~~ ~~analysis~~ is ~~useful~~ also beneficial in determining the operating conditions to achieve the desired conversion [30]. There are several experimental design models used in RSM, such as Box-Behnken Design (BBD), Control Composite Design (CCD) and Doehlert Design. The BBD has been broadly applied for biodiesel production optimization since it is effective, requires the smallest number of trials than the other designs [31]. In this ~~study~~investigation, analysis using BBD in RSM was performed to determine the best reaction operation condition which resulted in the highest reaction conversion ~~optimization~~ of the interesterification reaction of CSO in the presence of lipase enzyme catalyst ~~was performed using BBD in RSM analysis to determine the best reaction operation condition which resulted in the highest reaction conversion.~~within the ranges of the parameter values studied.

2. Materials and Methods

2.1. Materials

The materials used were: *Calophyllum inophyllum* seed oil (CSO) from UMKM Samtamanu Cilacap, Indonesia, ethyl acetate p.a (Merck), liquid lipase catalyst (Eversa Transform 2.0 from Novozyme), phosphoric acid p.a (Merck), KOH p.a (Merck), oxalic acid p.a (Merck), ethanol p.a (Merck), and aquadest from Indrasari chemicals store, Semarang, Indonesia. The catalyst used was liquid (free form) lipase Eversa Transform 2.0 (ET 2.0) from Novozyme, which is made from genetically modifed Aspergillus oryzae microorganism [32]. Commonly, lipase are active in broad range of temperature from 20-60 °C [33,34] and the ET 2.0 was reported to work satisfactorily up to the reaction temperature of 60°C [35].

2.2. Method

2.2.1. Degumming

Crude CSO as much as 100 mL was put into a 250 mL flat bottom flask. The oil was then heated using hot plate equipped with a magnetic stirrer at a speed of 600 rpm until the temperature reached 70°C. Phosphoric acid was subsequently added to the hot oil with the amount of 0.3% (w/w) of CSO and heated for 25 minutes. The oil was cooled at room temperature and put into a 500 mL separatory funnel. Distilled water at a temperature of 40°C was thereafter added with a volume of 5% of the CSO volume. Subsequent to the degumming process, the mixture was settled in the separatory funnel for 24 hours to attain the completion of the separation between the CSO and the residue. After the 24 hours decantation process, the two layers were appeared. The top layer of brownish yellow color was the refined CSO, while the dark brown bottom layer was gum, impurities, and water which should be separated. Let the mixture in the separatory funnel for to attain the completion of the separation between the oil and the residue. The degummed CSO was then separated from the residue and was heated in the oven with a temperature of 105°C to reduce the water content [30]. CSO was then analyzed to determine its physical properties (density, viscosity, and acid number).

2.2.2. Interesterification Reaction

After the degumming process, CSO was used as the raw material for biodiesel synthesis via interesterification reaction. CSO as much as 100 mL was introduced into a 250 mL flat bottom three neck flask and added with 10% (w/w) liquid lipase. The mixture was heated using a hot plate equipped with a magnetic stirrer with the stirring speed of 600 rpm until it reached the desired temperature. On the other hand, ethyl acetate was warmed up separately up to the similar temperature. When the reactants attained the specified temperature, ethyl acetate was subsequently poured into the reactor and mixed with the CSO and lipase. This incident was recorded as the reaction time of zero (t = 0 h). The ratio of CSO to ethyl acetate was fixed at 1:3. The stoichiometric ratio of the reactants was employed to investigate the optimum reaction conversion that can be obtained without any excess reactant (ethyl acetate) as it was conducted by Manurung et al. [36]. The reaction temperature was varied at 30°C, 40 °C, 50°C and 60°C.The reaction temperature was varied at 30°C, 40 °C, 50°C and 60°C. After the specified temperature was reached, the ethyl acetate with the similar temperature was added with the ratio of CSO to ethyl acetate of 1:3 (stoichiometric ratio). The reaction time was 1-5 h. Sample was taken periodically every 1 hour of the reaction time. Prior to the analysis, sample was centrifuged with the centrifugation speed of 40,000 rpm for 15 minutes to separate between biodiesel product and the catalyst residue. The sample analysis was carried out using Gas Chromatography-Mass Spectroscopy (GC-MS). The independent variables for the experimental work is shown in Table 1.

Table 1. Independent Variables.

Variable	Values
Reaction Time	1,2,3,4, and 5 hours
Reaction Temperature	30°C, 40°C, 50°C, and 60°C
Catalyst Concentration	10%, 15%, and 20%

2.2.3. Gas Chromatography-Mass Spectroscopy (GC-MS)

Analysis of the fatty acid composition of the biodiesel was determined using a Gas Chromatography-Mass Spectrometer (GC-MS QP2010 SE) with a column flow rate of 1.20 mL/min, an oven column temperature of 65°C, a pressure of 74.5 kPa, and an injecttion temperature of 250°C with split injection mode. The internal standard used was heptadecanoic acid, methyl ester.

2.2.4. Response Surface Methodology Analysis

The GC-MS data was utilized to determine the reaction conversion. The result was subsequently analyzed using Response Surface Methodology (RSM) with the Box Behnken Design (BBD) model on Design Expert software to determine the optimum operation condition. The BBD is a vigorous and extensively applied model for biodiesel synthesis optimization and parameters analysis [31]. The Levels of tested parameter for the BBD experiment are presented in Table 2.

Table 2. Levels of Tested Parameter for Box Behnken Design (BBD).

Independent Factor	Units	Symbol	Level	
			Low	High
Temperature	°C	A	30	50
Reaction Time	hours	B	1	5
Catalyst Concentration	%	C	10	20

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3. Results and Discussion

3.1. Effects of the Degumming Process on the CSO Properties

Naturally, the crude CSO contains phospholipids, sterols, free fatty acids, waxes, oil-soluble pigments and hydrocarbons. Degumming is the initial and essential refining process of crude vegetable oil to remove phospholipids and gums [37]. To date, there are various methods that can be employed to degum vegetable oil, explicitly water, acid, enzymatic, and membrane degumming techniques. Water degumming and acid degumming is the most used methods for vegetable degumming at industrial scale [38]. Those methods are simple, easy, and low cost. However, water degumming is less effective compared to the acid method. Gum comprises of the hydratable (HP) and non-hydratable phosphatides (NHP). HP can be eliminated using water. In contrast, NHP cannot be eradicated via water degumming [15,16]. Thus, acid degumming method is more advantageous in which it can remove both the HP and NHP. However, water degumming is less effective compared to the acid degumming. Thus, acid degumming method is more advantageous. The most used acid for degumming process are phosphoric or citric acid with the suggested concentration between 0.05 – 2% w/w oil [39]. In this work, 0.3% phosphoric acid was applied [23,40]. After the degumming process, the black color of the CSO turns into reddish yellow due to the loss of the phospholipids compound in crude CSO. Phospholipids can form dark color in vegetable oil as a result of the autoxidation process of these compounds during storage. Condensation reaction between the amino groups in phospholipids with aldehydes will yield melanophosphatides compound which give dark color to the oil [41]. Thus, removal of phospholipids in CSO will reduce the occurrence of the above mention reaction and diminish the color intensity.

Degumming process can improve the properties of CSO. In this work, the density, viscosity, and acid number of crude and degummed CSO were tested experimentally. The effects of the degumming process on the physicochemical properties of CSO is indicated in Table 3. It was obvious that the density and viscosity of the CSO were slightly decreasing after the degumming process due to the removal of gum and other impurities. The effect of degumming process on the CSO properties is indicated in Table 2. It was revealed that the density and viscosity of the CSO were slightly decreasing after the degumming process. It occurred to the removal of gum and other impurities. Besides, degumming also lead to the lessening of the acid number of the oil which was attributable to the decrease of the acid number and the free fatty acid (FFA) existence in the oil during the degumming process. This phenomenon is in line with the data reported by Adekunle et al.[42]. Fatty acid composition of the CSO after undergoing degumming process was presented in the Table 3, based on the interpretation of the chromatogram shown in Figure 1.

Table ~~2~~3. Effects of Degumming Process on the Properties of CSO.

CSO Properties	Before Degumming	After Degumming
Density (kg/m^3)	941	937
Viscosity (mm^2/s)	63.42	59.73
Acid Number (mg KOH/~~kg~~ CSO)	64.62	48.24
Free Fatty Acid Content (%)	32.47	24.25

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Table ~~3~~4. Fatty Acid Composition of the CSO after Degumming Process.

Fatty Acid	Molecular Weight (g/mol)	Area (%)
Palmitic Acid	256.22	7.82
Linoleic Acid	280.45	16.82
Oleic Acid	282.52	26.62
Stearic Acid	284.47	8.86
Arachidic Acid	312.54	0.31

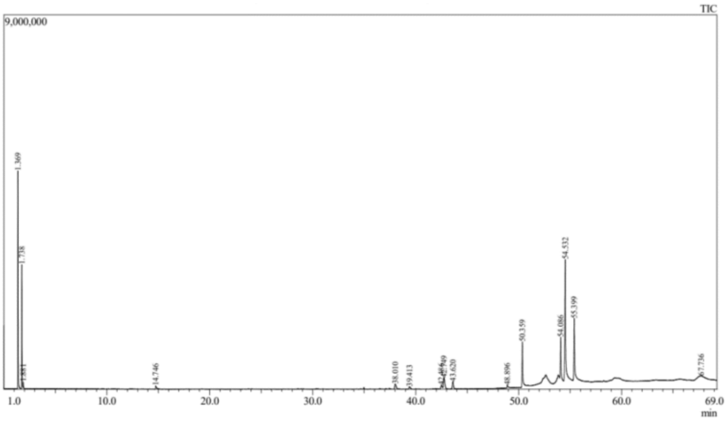


Figure 1. Chromatogram of the CSO after Degumming Process.

3.2. Effects of the Temperature on the Interesterification Reaction Conversion

After the degumming process, CSO directly underwent interesterification process with ethyl acetate in the presence of lipase catalyst. ~~A specific pre-treatment for reducing the FFA content of CSO was not considered necessary since the FFA presentation in the oil does not affect the enzymatic catalyzed reaction. The influences of the reaction time and temperature on the reaction conversion were observed at the fixed lipase catalyst concentration of 10% (w/w). The result is exhibited in Figure 2.~~ As depicted in Table 2, the crude and degummed CSO contained high FFA of 32.47% and 24.25%, respectively, which were over 2 mgKOH/g. However, enzymatic reaction is generally insensitive to the existence of the FFA impurities in the oil feedstock. The possibility of the undesired saponification reaction can be neglected. Therefore, a specific pre-treatment for reducing the FFA content of CSO is considered unnecessary since the FFA presentation in the oil does not affect the enzymatic catalyzed reaction [22,43].

The influences of the reaction time and temperature on the reaction conversion were observed at the fixed lipase catalyst concentration of 10% (w/w). The result is exhibited in Figure 2. Basically, lipase are active in the temperature range of 20-60 °C [33,34] and the ET 2.0 lipase can work up to 60°C [35]. Moreover, the reaction time is often studied up to 12 h, but the significant reaction rate is in the range of 0 – 5 h [44]. In this work,~~Based on the experimental data,~~ it was disclosed that the highest reaction conversion was 54.99%, obtained at the reaction temperature of 40°C and the reaction time of 3 hours. Commonly, the reaction conversion is enhanced with the increasing of the reaction temperature and reaction time since at the higher temperature, the viscosity of the liquid decreases, causing the higher solubility of reactants, mass diffusion, rate of reaction, and conversion [45]. However, in this work, the reaction conversion decreased when the reaction was performed at 50°C for 5 hours. This trend was in accordance with the result recorded by Gusniah et al. [46]. It was reported that the lipase enzyme catalyst activity in the transesterification reaction of waste cooking oil reached the optimum performance at 40 °C and it declined to some extent when the temperature rose to 50 °C. It happened since the lipase catalyst began to denature, causing the damage of the active part of the enzyme at the higher temperature. Beyond the optimum temperature, lipase catalyst becomes unstable and misplaces its tertiary structure which causes the shortfall of its activity [45]. It thus leads to the declining of the reaction rate and conversion. Generally, the lipase enzyme reached the highest activity at the temperature of 40°C – 45°C as stated by Murtius et al. [47], Ayinla et al. [48], and Yazid [49].

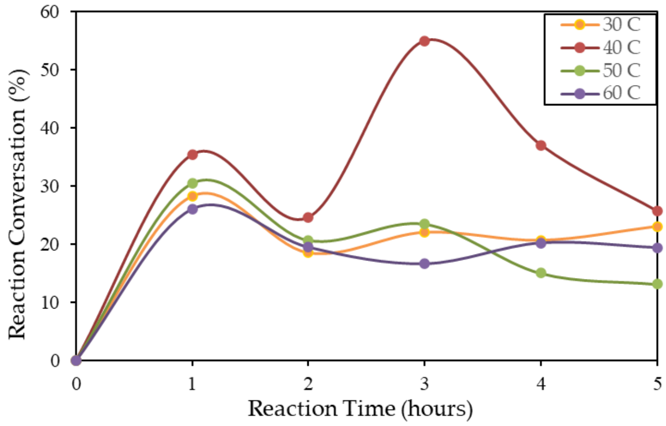


Figure 2. Effects of the reaction time and temperature on the reaction conversion at fixed lipase catalyst concentration of 10% (w/w).

3.3. *Effects of the Catalyst Concentration e on the Interesterification Reaction Conversion*

Investigation on the influence of the lipase catalyst concentration was carried out at the molar ratio of oil to ethyl acetate of 1:3, reaction temperature of 40°C and reaction time of 5 hours. The lipase concentration studied was 10%, 15%, and 20%. Figure 3 has shown that the optimum catalyst concentration was 15%, resulting in the reaction conversion of 28.445%. This result was in agreement with the resume conveyed by Gusniah et al. [46] which conveyed that the highest lipase catalyst loading was 15%. However, the reaction conversion in this work was still low compared to the other research. It came about since the reaction was conducted with the low molar ratio of oil and ethyl acetate (1:3) and short reaction time (5 h). As comparison, Sun et al. [44] achieved the 94.2% yield for the transesterification of semen Abutili seed oil in the presence of a similar lipase catalyst but at the far excess molar ratio of oil and alcohol (1:7) and longer reaction time (11 h).

Subhedar and Gogate [29] carried out the interesterification reaction of waste cooking oil and methyl acetate using immobilized lipase obtained from *Thermomyces lanuginosus* (Lipozyme TLIM) as a catalyst. The optimum were demonstrated at the oil : methyl acetate molar ratio of 1:12, enzyme concentration of 6% (w/v), reaction temperature of 40 °C and reaction time of 24 h. Biodiesel yield was 90.1% under these operation conditions, which was higher than this work. The higher reaction conversion can be provided since the reaction was conducted on an excessive molar ratio and extensively longer reaction time (24 h). Besides, this reaction employed an immobilized lipase, in which the lipase was attached on the carrier substrate, which led to the higher product purity. Thus, it can be deduced that the lipase catalyzed reaction this work resulted in a lower conversion due to a shorter reaction time and low molar ratio of the reactants, which was not excess than the stoichiometric ratio. Besides that, utilization of the liquid lipase can reduce the apparent concentration of biodiesel yield since it can act as an impurity in the final biodiesel product.

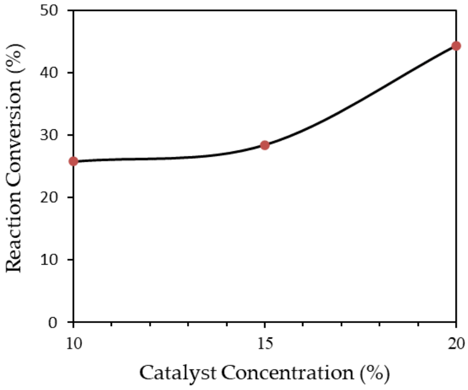


Figure 3. Effects of the Lipase Catalyst Concentration at the Reaction Temperature of 40°C and Reaction Time of 5 Hours.

3.4. *Response Surface Methodology (RSM) Analysis*

Response surface methodology (RSM) is the broadest employed method of experimental design for optimization. This technique applies mathematical and statistical method for modeling and analyzing a process which involves numerous parameters. The purpose of the RSM is to optimize the response variables [29]. The factors that influence the process are entitled dependent variables. Meanwhile, the responses are named as dependent variables. Therefore, analysis using ~~Response Surface Methodology (~~RSM~~)~~ is useful in designing operating conditions to achieve the targeted conversion. Many literatures have shown that the most applicable tools in RSM for optimization of biodiesel production process are the Box Behnken Design (BBD) and Central Composite Design (CCD) tools [50]. BBD is favored than CCD since it provides higher efficiency but more economical [51,52]. To evade the failure and extreme reaction variables value, BBD is limited to three levels. Hence, BBD typically has no factorial points. The BBD tool was also utilized by Sharma et al. [53] and Rokni et al. [54].

In this study, BBD was applied to obtain the ~~optimal~~ best operating conditions and highest conversion for biodiesel synthesis via interesterification reaction within the ranges of the parameter values studied. The Box Behnken Design (BBD) tool in RSM was used to evaluate the effect of the independent variables (reaction time, reaction temperature, and catalyst concentration). The experimental data of the reaction conversion compared with the prediction using BBD is exhibited in Table ~~4~~5.

The parameters used to obtain the appropriate model to predict the response results must match the observed model to the experimental data. Analysis of variance (ANOVA) was accomplished using the most common types of polynomial models, namely linear, interactive (2FI), quadratic and cubic can be applied to predict the response variables of experimental data. Several parameters such as sequential p-value, lack of fit p-value, adjusted R¬2, predicted R2 and Adiq precision were used to conclude the most suitable type of model for the optimization of the conversion of biodiesel produced from CSO. In this study, optimization using RSM was initially performed using the quadratic model. The ANOVA quadratic model is presented in Table ~~5~~6.

Table ~~4~~5. Experimental Data of the Reaction Conversion and the Prediction Using BBD.

Run	Temperature, °C	Reaction Time, hr	Catalyst Concentration, % w/w	Reaction Conversion, % Experiment	Reaction Conversion, % Prediction	Error, %
1	40	3	15	12.92	18.15	40.4
2	30	3	10	22.08	19.877	9.97
3	40	5	20	48.92	48.03	1.8
4	30	3	20	27.75	27.33	1,5
5	40	5	10	25.77	26.66	3.4
6	40	3	15	18.27	18.15	0.6
7	30	1	15	23.41	24.72	5.6
8	50	5	15	14.90	13.59	8.8
9	40	1	20	13.55	12,66	6.5
10	30	5	15	22.10	23.41	5.9
11	40	3	15	23.25	18.15	21.9
12	40	1	10	35.43	36.32	2.5
13	50	1	15	26.82	25.51	4.8
14	50	3	20	12.01	14.22	18.3
15	50	3	10	23.53	23.96	1.7

Table ~~5~~6. ANOVA of the Quadratic Model.

Source	Sum of Squares	DF	Mean Square	F-value	p-value	
Model	1007.96	9	112.00	2.13	0.2098	not significant
A-Suhu	40.83	1	40.83	0.7760	0.4187	
B-Waktu	19.45	1	19.45	0.3696	0.5698	
C-Katalis	2.61	1	2.61	0.0497	0.8325	
AB	28.14	1	28.14	0.5348	0.4974	
AC	73.90	1	73.90	1.40	0.2892	
BC	507.01	1	507.01	9.64	0.0267	
A^2	32.27	1	32.27	0.6134	0.4690	
B^2	161.73	1	161.73	3.07	0.1399	
C^2	139.90	1	139.90	2.66	0.1639	
Residual	263.06	5	52.61			
Lack of Fit	209.74	3	69.91	2.62	0.2881	not significant
Pure Error	53.32	2	26.66			
Cor Total	1271.02	14				

Based on the quadratic model, it was revealed that the p-value was 0.~~2998~~2098, which did not meet the requirement of the p-value < 0.05 as demonstrated in Tabel ~~5~~6. Hence, the quadratic model was not significant for this case. To overcome this obstacle, modified model was developed to obtain the significant model [55]. Modification of the model shown that the reduced cubic model made evident of the significant model (Table ~~6~~7).

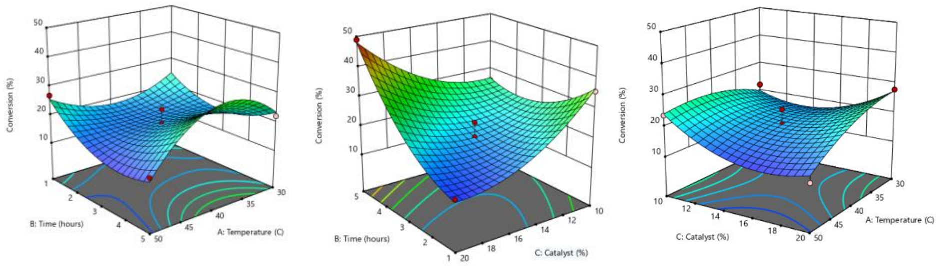
Table 6. ANOVA of the Reduced Cubic Model.

Source	Sum of Squares	DF	Mean Square	F-value	p-value	
Model	1197.58	10	119.76	6.52	0.0428	significant
A-Suhu	40.83	1	40.83	2.22	0.2102	
B-Waktu	165.26	1	165.26	9.00	0.0399	
C-Katalis	2.61	1	2.61	0.1423	0.7252	
AB	28.14	1	28.14	1.53	0.2834	
AC	73.90	1	73.90	4.03	0.1153	
BC	507.01	1	507.01	27.62	0.0063	
A^2	32.27	1	32.27	1.76	0.2555	
B^2	161.73	1	161.73	8.81	0.0412	
C^2	139.90	1	139.90	7.62	0.0508	
A^2B	189.62	1	189.62	10.33	0.0325	
Residual	73.44	4	18.36			
Lack of Fit	20.12	2	10.06	0.3773	0.7260	not significant
Pure Error	53.32	2	26.66			
Cor Total	1271.02	14				
Adeq Precision	9.6401					

It was found that the p-value of the modified cubic model was 0.0428, which has met the requirement of p-value > 0.05. Furthermore, the value of lack-of-fit was examined. Lack-of-fit is a test that analyzes how satisfactory the full models suit with the data. Model with a significant lack-of-fit should not be applied for the prediction. In this work, the lack-of-fit was not significant, which means that the model can be employed for the prediction. The value of adeq precision is the measure of the ratio of the signal to the disturbance. The expected value of the ratio is > 4 [50]. This model provided the value of the Adeq precision of 9.6401, indicating that the model is proper. Therefore, the reduced cubic model can be used to describe the response on the reaction conversion. The empirical correlation of the reduced cubic model in the form of polynomial orde for the reaction conversion in the CSO esterification is displayed in Equation ~~1~~3.

$$\text{Conversion (\%)} = 18.15 - 2.26\ A + 6.43\ B - 0{,}5715\ C - 2.65\ AB - 4.30\ AC + 11.26\ BC - 2.96\ A^2 + 6.62\ B^2 + 6.16\ C^2 - 9.74\ A^2B \quad (1)$$

Where, A is the reaction temperature (^{o}C), B is the reaction time (min) and C is the Catalyst Concentration (%). Based on the reduced cubic model, analysis on the effects and the process variables interaction on the response variable were carried out using 3D RSM graphs. The results are presented in Figure 4.



(a) (b) (c)

Figure 4. The 3D RSM graph representing the effects of reaction time, temperature and catalyst concentration on the reaction conversion.

Figure 4(a) shows the interaction between temperature and reaction time which gives a significant change to the increase in conversion. It was found that there was an increase in conversion at the temperature of 40°C and reaction time of 5 hours. This phenomenon is in line with the investigation of Handayani et al. [55], which reported that the optimum conversion was obtained at the temperature of 45°C and reaction time of 6 hours. Figure 4(b) discloses that the longer the reaction time employed, the higher the conversion achieved. It happened since the reaction time is directly proportional to the amount of product yielded. Li et al. [56] conveyed that the reaction conversion of *Pseudomonas cepacia* lipase was low at the reaction time of 3 h. (44%). On the contrary, it rose to 73% when the reaction time extended to 12 h. Figure 4(c) bares that the lower the reaction temperature applied, the higher conversion resulted in. It occurred since the lipase enzyme activity works at the temperature of 30°C – 45°C. In the enzymatic reaction, the increase of the temperature will promote the reaction rate and accordingly the reaction conversion due to the decrease of the viscosity which enhance the solubility of oil and methanol. However, at a certain temperature, reaction conversion declines evoking the enzyme deactivation [57]. Meanwhile, the addition of the catalyst concentration increased the reaction conversion. This result was in line with the description of Gusniah et al. [46]. It was justified that the increasing amount of the enzyme employed will provide a higher amount of the available active sites for the reaction, leading to the higher reaction conversion.

The Derringer method is utilized to optimize the response which is characterized by the presence of a desirability function. Desirability shows how close the optimization results are to the optimum point. The desirability function (DF) is constructed on the transformation of all the acquired responses from different scales into a scale-free value. The values of DF are ranging from 0 to 1. The value 0 means that the factors provide an undesirable response. On the other hand, the value 1 denotes the optimal performance of the evaluated factor [58]. Hence, the desirability value which is close to 1 is the expected value. However, the RSM analysis is not only to find the desirability value which is equal to 1, but to determine the conditions that match expectations. The multiple correlation coefficient (R^2) was closed to 1. It denoted that the models have good predictive ability. Based on the ~~optimization~~ RSM analysis results, the ~~optimum~~ highest reaction conversion was 41.46%, attained at a temperature of 44.43°C, a reaction time of 5 hours and a catalyst concentration of 20%, with a desirability value of 0.733. This finding can be stated as the local optimum within the certain parameter range studied, which is worthwhile as a basis for developing process intensification in order to obtain a higher conversion.

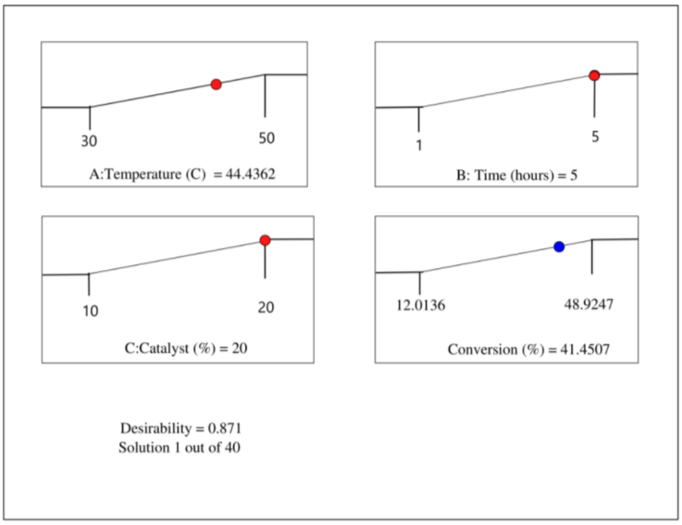


Figure 7. The Highest Reaction Conversion Based on the ~~Optimization Using~~ RSM Analysis.

5. Conclusions

Based on the RSM analysis, it was revealed that reaction time and catalyst concentration had significant effects on the conversion of the interesterification reaction of CSO with ethyl acetate in the presence of lipase catalyst for biodiesel synthesis. The results of the analysis showed that the ~~optimum~~ highest reaction conversion was 41.46% at the reaction temperature, reaction time, and catalyst concentration of 44.40°C, 5 hours, and 20%, respectively, which can be stated as the local optimum. The result was good, nonetheless, it is not economically feasible yet for industrial scale production and purpose with the current condition. Therefore, the further process intensification is necessary to enhance the reaction conversion and biodiesel yield.

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Authors: Ratna Dewi Kusumaningtyas *, Normaliza Normaliza, Elva Dianis Novia Anisa, Haniif Prasetiawan, Dhoni Hartanto, Harumi Veny, Fazlena Hamzah, Miradatul Najwa Muhd Rodhi
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Article

Synthesis of Biodiesel via Interesterification Reaction of *Calophyllum inophyllum* Seed Oil and Ethyl Acetate over Lipase Catalyst: Experimental and Surface Response Methodology Analysis

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Abstract: Biodiesel is increasingly being considered as an alternative to the fossil fuel as it is renewable, nontoxic, biodegradable, and feasible for mass production. Biodiesel can be produced from various types of vegetable oils. *Calophyllum inophyllum* seed oil (CSO) is among the prospective nonedible vegetable oils considered as a raw material for biodiesel synthesis. The most common process of the biodiesel manufacturing is the transesterification of vegetable oils which results in glycerol as a by-product. Thus, product purification is necessary. In this work, an alternative route to biodiesel synthesis through interesterification reaction of vegetable oil and ethyl acetate was conducted. By replacing alcohol with ethyl acetate, triacetin was produced as a side product rather than glycerol. Triacetin can be used as a fuel additive to increase the octane number of the fuel. Therefore, triacetin separation from biodiesel products is needless. The interesterification reaction is catalyzed by an alkaline catalyst or by a lipase enzyme. In this study, biodiesel synthesis was carried out using a lipase enzyme since it is a green and sustainable catalyst. The interesterification reaction of CSO with ethyl acetate in the presence of a lipase catalyst was conducted using the molar ratio of CSO and ethyl acetate of 1:3. The reaction time, lipase catalyst concentration, and reaction temperature were varied at 1, 2, 3, 4, 5 h, 10%,15%, 20%, and 30 °C, 40 °C, 50 °C, 60 °C, respectively. The experimental results were also analyzed using response surface methodology (RSM) with the Box–Behnken design (BBD) model on Design Expert software. Data processing using RSM revealed that the highest conversion within the studied parameter range was 41.46%, obtained at a temperature reaction of 44.43 °C, a reaction time of 5 h, and a lipase catalyst concentration of 20%.

Keywords: biodiesel; *Calophyllum inophyllum* seed oil; interesterification; enzymatic; Box–Behnken design

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1. Introduction

Biodiesel is an alternative fuel for diesel engines which is synthesized from vegetable oils or animal fats. Biodiesel is one of the modern bioenergies which has several advantages. It has non-toxic properties, low emission rates and no sulfur gas, just to name a few. Thus, biodiesel is a prospective alternative as an environmentally friendly diesel

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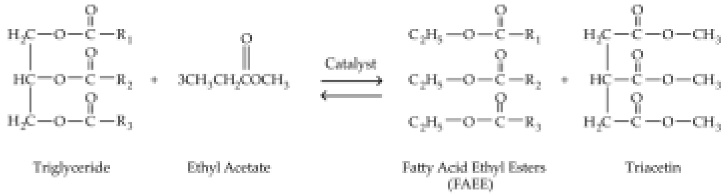
fuel [1]. Biodiesel is commonly derived from vegetable oil which has renewable characteristics. The main components of vegetable oils and animal fats are triglycerides, which can be converted into mono alkyl esters of long chain fatty acid or termed as fatty acid alkyl ester (biodiesel). Various types of vegetable oils can be applied as prospective raw materials for biodiesel preparation [2]. The widely used raw material for biodiesel synthesis in Indonesia is crude palm oil (CPO). CPO is abundantly available since it is produced on a large scale. However, the production of palm oil biodiesel competes with food needs. Therefore, other alternative vegetable oils are needed as raw materials for biodiesel. Nonedible oil feedstocks are favorable to ensure the sustainability biodiesel production [3,4].

There are several nonedible oils that have been widely investigated as biodiesel feedstocks, such as silk–cotton or *Ceiba pentandra* seed oil [5], jatropha oil [4,6], castor oil [7], rubber seed oil [8], karanja oil [9], mahua oil, neem oil [10], waste cooking oil [11], palm fatty acid distillate [12] and *Calophyllum inophyllum* seed oil [13]. One among the potential nonedible vegetable oils in Indonesia is *Calopyllum inophyllum* seed oil (CSO). It is also known as tamanu or nyamplung seed oil. Akram et al. [1] reported that *Calopyllum inophyllum* (tamanu) seed has an oil content of 65–75%, which is higher than other nonedible seed oil plants, such as jatropha seed oil (27–40%), rubber seed kernel oil (40–50%), and castor oil (45–65%). Rasyid et al. [14] reported that the fatty acids composition of CSO comprises of oleic acid, palmitic acid, linoleic acid, and stearic acid. According to Adenuga et al. [13], CSO biodiesel meets the Australian, ASTM and EN standards. Therefore, CSO is suitable to be used as a feedstock in the production of biodiesel. However, CSO contains high gummy substances, especially in the form of lipoid A (the hydratable phosphatides) and lipoid B (the non-hydratable phosphatides [15,16], waxes, and other impurities [17]. The existence of gum is unfavorable since it may lower the reaction rate which in consequence reduces the biodiesel yield. At an industrial level, this condition leads to an economic disadvantage. Besides, the presence of phosphorus compounds can generate the carbon particle deposition in diesel engine which causes plugging in the engine filter, line, and injectors, as well as reducing engine performance [17,18]. Thus, a degumming process is essential as the pretreatment step of biodiesel feedstocks with a high phosphorus content [19].

The most general method for making biodiesel is an alkaline-catalyzed transesterification reaction by reacting vegetable oils with short-chain alcohols such as methanol or ethanol [20,21]. However, the transesterification reaction has a limitation in terms of the potential for a saponification side reaction if the oil feedstock contains high free fatty acid (FFA) 2 mgKOH/g, as described by Kusumaningtyas et al. [4] and Sebastian et al. [22]. Besides, the transesterification reaction results in glycerol as a by-product, which needs a separation process to obtain high purity biodiesel. Biodiesel purification from glycerol by-products requires a series of separation steps which brings in high operation costs. To overcome these problems, it is necessary to apply the process of producing biodiesel which is glycerol-free through an interesterification reaction [23]. In the interesterification reaction, the use of methanol or ethanol is replaced with methyl acetate or ethyl acetate. Interesterification of triglyceride yields a triacetin co-product instead of glycerol [24]. Triacetin has an advantage as a fuel additive, a good anti-knocking, and can enhance the octane number. The addition of triacetin to the fuel also offers an environmental benefit since it reduces the exhaust smoke and NO emissions to some extent. It can be stated that the existence of the triacetin co-product in biodiesel contributes to engine performance improvement [25], as the addition of triacetin increases the oxygen content of the fuel [26]. Thus, it is unnecessary to separate triacetin from biodiesel products. A comparison between transesterification and interesterification reaction schemes are presented in Equations (1) and (2), respectively.



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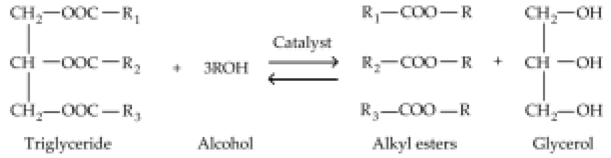

(2)

To date, the development of green energy through environmentally friendly processes has become a priority. One alternative towards a greener process is reducing the use of chemical catalysts and switching to enzymatic catalysts (biocatalysts). The enzyme-catalyzed biodiesel synthesis shows superiority compared to the chemically catalyzed one, mainly in terms of the lower energy requirement and natural conservation aspect. Lipase is the most common enzyme employed for biodiesel synthesis. There are two principal classes of lipase, free enzymes and immobilized enzymes. Free lipase enzymes are cheaper and simpler, thus it is feasible for large-scale applications [27]. Besides that, the use of lipase enzymes is very promising to overcome the disadvantages of alkaline catalysts related to the occurrence of the undesired saponification reaction when high FFA oil used as raw material. Lipase enzyme catalysts can be applied with high FFA feedstocks without any necessity of FFA removal as a pretreatment step. However, lipase biocatalysts are easily deactivated in an alcoholic environment since short-chain alcohols often cause irreversible loss of enzyme activity [28]. Therefore, the non-alcoholic interesterification route is preferable in order to maintain high biocatalyst activity and stability during the reaction. In the interesterification reaction, alcohol can be replaced by methyl acetate or ethyl acetate, which is harmless for the lipase catalyst [29].

In this work, biodiesel was synthesized through lipase-catalyzed interesterification of *Calopyllum inophyllum* seed oil (CSO) with ethyl acetate. The lipase enzyme used was the liquid-free lipase from Novozyme. The effects of the main parameters on the reaction conversion were studied experimentally. Analysis using the response surface methodology was also carried out to investigate the best reaction conversion obtained within the values range of the studied independent variables. Process optimization was also conducted using the response surface methodology, a collection of mathematical and statistical calculation techniques based on the compatibility of the empirical model with the experimental data obtained. Generally, RSM is useful for the modeling and analysis of problems that influences the response variables with the purpose of optimizing the response. RSM is also beneficial when determining the operating conditions to achieve the desired conversion [30]. There are several experimental design models used in RSM, such as the Box–Behnken Design (BBD), control composite design (CCD) and Doehlert Design. The BBD has been broadly applied for biodiesel production optimization since it is effective, requires the smallest number of trials than the other designs [31]. In this investigation, analysis using BBD in RSM was performed to determine the best reaction operation condition which resulted in the highest reaction conversion of the interesterification reaction of CSO in the presence of lipase enzyme catalyst within the ranges of the parameter values studied.

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2. Materials and Methods

2.1. Materials

The materials used were: *Calophyllum inophyllum* seed oil (CSO) from UMKM Samtamanu Cilacap, Indonesia, ethyl acetate p.a (Merck), liquid lipase catalyst (Eversa Transform 2.0 from Novozyme*)*, phosphoric acid p.a (Merck), KOH p.a (Merck), oxalic acid p.a (Merck), ethanol p.a (Merck), and aquadest from Indrasari chemicals store, Semarang, Indonesia. The catalyst used was liquid (free form) lipase Eversa Transform 2.0 (ET 2.0) from Novozyme, which is made from genetically modifed Aspergillus oryzae microorganism [32]. Commonly, lipase are active in broad range of temperature from 20–60 °C [33,34] and the ET 2.0 was reported to work satisfactorily up to the reaction temperature of 60 °C [35].

2.2. Method

2.2.1. Degumming

Crude CSO as much as 100 mL was put into a 250 mL flat bottom flask. The oil was then heated using hot plate equipped with a magnetic stirrer at a speed of 600 rpm until the temperature reached 70 °C. Phosphoric acid was subsequently added to the hot oil with the amount of 0.3% (*w/w*) of CSO and heated for 25 min. The oil was cooled at room temperature and put into a 500 mL separatory funnel. Distilled water at a temperature of 40 °C was thereafter added with a volume of 5% of the CSO volume. Subsequent to the degumming process, the mixture was settled in the separatory funnel for 24 h to attain the completion of the separation between the CSO and the residue. After the 24 h decantation process, the two layers were appeared. The top layer of brownish yellow color was the refined CSO, while the dark brown bottom layer was gum, impurities, and water which should be separated. Let the mixture in the separatory funnel for to attain the completion of the separation between the oil and the residue. The degummed CSO was then separated from the residue and was heated in the oven with a temperature of 105 °C to reduce the water content [30]. CSO was then analyzed to determine its physical properties (density, viscosity, and acid number).

Comment [M7]: We added the italics. Please confirm this revision.

Response:
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2.2.2. Interesterification Reaction

After the degumming process, CSO was used as the raw material for biodiesel synthesis via interesterification reaction. CSO as much as 100 mL was introduced into a 250 mL flat bottom three neck flask and added with 10% (*w/w*) liquid lipase. The mixture was heated using a hot plate equipped with a magnetic stirrer with the stirring speed of 600 rpm until it reached the desired temperature. On the other hand, ethyl acetate was warmed up separately up to the similar temperature. When the reactants attained the specified temperature, ethyl acetate was subsequently poured into the reactor and mixed with the CSO and lipase. This incident was recorded as the reaction time of zero (t = 0 h). The ratio of CSO to ethyl acetate was fixed at 1:3. The stoichiometric ratio of the reactants was employed to investigate the optimum reaction conversion that can be obtained without any excess reactant (ethyl acetate) as it was conducted by Manurung et al. [36]. The reaction temperature was varied at 30 °C, 40 °C, 50 °C and 60 °C.The reaction temperature was varied at 30 °C, 40 °C, 50 °C and 60 °C. After the specified temperature was reached, the ethyl acetate with the similar temperature was added with the ratio of CSO to ethyl acetate of 1:3 (stoichiometric ratio). The reaction time was 1–5 h. Sample was taken periodically every 1 h of the reaction time. Prior to the analysis, sample was centrifuged with the centrifugation speed of 40,000 rpm for 15 min to separate between biodiesel product and the catalyst residue. The sample analysis was carried out using Gas Chromatography-Mass Spectroscopy (GC-MS). The independent variables for the experimental work is shown in Table 1.

Table 1. Independent Variables.

Variable	Values
Reaction Time	1, 2, 3, 4, and 5 h
Reaction Temperature	30 °C, 40 °C, 50 °C, and 60 °C
Catalyst Concentration	10%, 15%, and 20%

2.2.3. Gas Chromatography-Mass Spectroscopy (GC-MS)

Analysis of the fatty acid composition of the biodiesel was determined using a Gas Chromatography-Mass Spectrometer (GC-MS QP2010 SE) with a column flow rate of 1.20 mL/min, an oven column temperature of 65 °C, a pressure of 74.5 kPa, and an injecttion temperature of 250 °C with split injection mode. The internal standard used was heptadecanoic acid, methyl ester.

2.2.4. Response Surface Methodology Analysis

The GC-MS data was utilized to determine the reaction conversion. The results were subsequently analyzed using response surface methodology (RSM) with the Box–Behnken Design (BBD) model on Design Expert software to determine the optimum operation condition. The BBD is a vigorous and extensively applied model for biodiesel synthesis optimization and parameter analysis [31]. The levels of the tested parameters for the BBD experiment are presented in Table 2.

Table 2. Levels of Tested Parameters for Box–Behnken Design (BBD).

Independent Factor	Units	Symbol	Level	
			Low	High
Temperature	°C	A	30	50
Reaction Time	hours	B	1	5
Catalyst Concentration	%	C	10	20

3. Results and Discussion

3.1. Effects of the Degumming Process on the CSO Properties

Naturally, crude CSO contains phospholipids, sterols, free fatty acids, waxes, oil-soluble pigments and hydrocarbons. Degumming is the initial and essential refining process of crude vegetable oil to remove phospholipids and gums [37]. To date, there are various methods that can be employed to degum vegetable oil, explicitly water, acid, enzymatic, and membrane degumming techniques. Water degumming and acid degumming are the most used methods for vegetable degumming at an industrial scale [38]. Those methods are simple, easy, and low cost. However, water degumming is less effective compared to the acid method. The gum is comprised of hydratable (HP) and non-hydratable phosphatides (NHP). HP can be eliminated using water. In contrast, NHP cannot be eradicated via water degumming [15,16]. Thus, the acid degumming method is more advantageous in that it can remove both the HP and NHP. However, water degumming is less effective compared to acid degumming. Thus, the acid degumming method is more advantageous. The most used acids for the degumming process are phosphoric or citric acid, with a suggested concentration between 0.05–2% *w/w* oil [39]. In this work, 0.3% phosphoric acid was applied [23,40]. After the degumming process, the black color of the CSO turns reddish-yellow due to the loss of the phospholipids compound in crude CSO. Phospholipids can form dark colors in vegetable oil as a result of the autoxidation process of these compounds during storage. The condensation reaction between the amino groups in phospholipids with aldehydes will yield melanophosphatides compounds which give a dark color to the oil [41]. Thus, removal of phospholipids in CSO will reduce the occurrence of the abovementioned reaction and diminish the color intensity.

The degumming process can improve the properties of CSO. In this work, the density, viscosity, and acid number of crude and degummed CSO were tested experimentally. The effects of the degumming process on the physicochemical properties of CSO is indicated in Table 3. It was obvious that the density and viscosity of the CSO were slightly decreasing after the degumming process due to the removal of gum and other impurities. The effect of the degumming process on the CSO properties is indicated in Table 2. It was revealed that the density and viscosity of the CSO slightly decreased after the degumming process. It occurred with the removal of gum and other impurities. Besides, degumming also leads to a lessening of the acid number of the oil which is attributable to the decrease of the acid number and the existence of free fatty acids (FFA) in the oil during the degumming process. This phenomenon is in line with the data reported by Adekunle et al. [42]. The fatty acid composition of the CSO after undergoing the degumming process is presented in the Table ~~3~~4, based on the interpretation of the chromatogram shown in Figure 1.

Table 3. Effects of Degumming Process on the Properties of CSO.

CSO Properties	Before Degumming	After Degumming
Density (kg/m^3)	941	937
Viscosity (mm^2/s)	63.42	59.73
Acid Number (mg KOH/g CSO)	64.62	48.24
Free Fatty Acid Content (%)	32.47	24.25

Table 4. Fatty Acid Composition of the CSO after Degumming Process.

Fatty Acid	Molecular Weight (g/mol)	Area (%)
Palmitic Acid	256.22	7.82
Linoleic Acid	280.45	16.82
Oleic Acid	282.52	26.62
Stearic Acid	284.47	8.86
Arachidic Acid	312.54	0.31

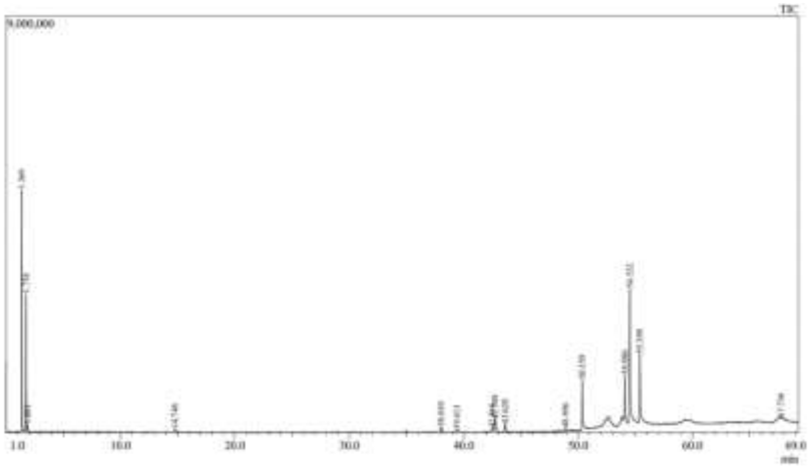

Figure 1. Chromatogram of the CSO after Degumming Process.

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Response:
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“The fatty acid composition of the CSO after undergoing the degumming process is presented in the Table 4, based on the interpretation of the chromatogram shown in Figure 1.”

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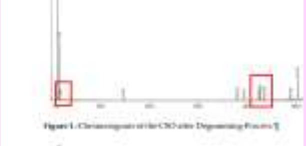

3.2. Effects of the Temperature on the Interesterification Reaction Conversion

After the degumming process, CSO directly underwent the interesterification process with ethyl acetate in the presence of the lipase catalyst. As depicted in Table 2, the crude and degummed CSO contained high FFA of 32.47% and 24.25%, respectively, which were over 2 mgKOH/g. However, the enzymatic reaction is generally insensitive to the existence of the FFA impurities in the oil feedstock. The possibility of the undesired saponification reaction can be neglected. Therefore, a specific pre-treatment for reducing the FFA content of CSO was considered unnecessary since the FFA presentation in the oil does not affect the enzymatic catalyzed reaction [22,43].

The influences of the reaction time and temperature on the reaction conversion were observed at a fixed lipase catalyst concentration of 10% (*w*/*w*). The result is exhibited in Figure 2. Basically, lipases are active in a temperature range of 20–60 °C [33,34] and the ET 2.0 lipase can work up to 60℃ [35]. Moreover, the reaction time is often studied for up to 12 h, but the significant reaction rate is in the range of 0–5 h [44]. In this work, it was disclosed that the highest reaction conversion was 54.99%, obtained at a reaction temperature of 40 °C and a reaction time of 3 h. Commonly, the reaction conversion is enhanced with the increasing of the reaction temperature and reaction time, since at the higher temperature the viscosity of the liquid decreases, causing the higher solubility of reactants, mass diffusion, rate of reaction, and conversion [45]. However, in this work, the reaction conversion decreased when the reaction was performed at 50 °C for 5 h. This trend was in accordance with the results recorded by Gusniah et al. [46], where it was reported that the lipase enzyme catalyst activity in the transesterification reaction of waste cooking oil reached an optimum performance at 40 °C and it declined to some extent when the temperature rose to 50 °C, which happened when the lipase catalyst began to denature, causing damage of the active part of the enzyme at the higher temperature. Beyond the optimum temperature, the lipase catalyst becomes unstable and misplaces its tertiary structure, which causes the shortfall in its activity [45]. It thus leads to the decline of the reaction rate and conversion. Generally, the lipase enzyme reached the highest activity at the temperature of 40–45 °C as stated by Murtius et al. [47], Ayinla et al. [48], and Yazid [49].

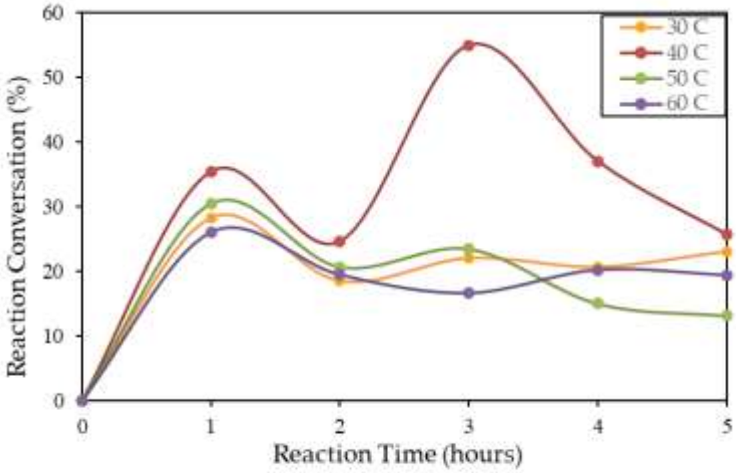

Figure 2. Effects of the reaction time and temperature on the reaction conversion at a fixed lipase catalyst concentration of 10% (*w*/*w*).

3.3. Effects of the Catalyst Concentration E on the Interesterification Reaction Conversion

Investigation on the influence of the lipase catalyst concentration was carried out at the molar ratio of oil to ethyl acetate of 1:3, a reaction temperature of 40 °C and a reaction time of 5 h. The lipase concentrations studied were 10%, 15%, and 20%. Figure 3 has shown that the optimum catalyst concentration was 15%, resulting in the reaction conversion of 28.445%. This result was in agreement with the results obtained by Gusniah et al. [46] which indicated that the highest lipase catalyst loading was 15%. However, the reaction conversion in this work was still low compared to those obtained in other research as the reaction was conducted with the low molar ratio of oil and ethyl acetate (1:3) and a short reaction time (5 h). By comparison, Sun et al. [44] achieved a 94.2% yield for the transesterification of semen Abutili seed oil in the presence of a similar lipase catalyst but at an excess molar ratio of oil and alcohol (1:7) and a longer reaction time (11 h).

Subhedar and Gogate [29] carried out the interesterification reaction of waste cooking oil and methyl acetate using immobilized lipase obtained from *Thermomyces lanuginosus* (Lipozyme TLIM) as a catalyst. The optimum reaction was demonstrated at the oil: methyl acetate molar ratio of 1:12, enzyme concentration of 6% (*w/v*), reaction temperature of 40 °C and reaction time of 24 h. Biodiesel yield was 90.1% under these operation conditions, which was higher than the results obtained in this work. A higher reaction conversion was obtained as the reaction was conducted on an excessive molar ratio and an extensively longer reaction time (24 h). Besides, this reaction employed an immobilized lipase, in which the lipase was attached on the carrier substrate, which led to higher product purity. Thus, it can be deduced that the lipase-catalyzed reaction in this work resulted in a lower conversion due to a shorter reaction time and low molar ratio of the reactants, which did not exceed the stoichiometric ratio. Besides that, utilization of the liquid lipase can reduce the apparent concentration of biodiesel yield since it can act as an impurity in the final biodiesel product.

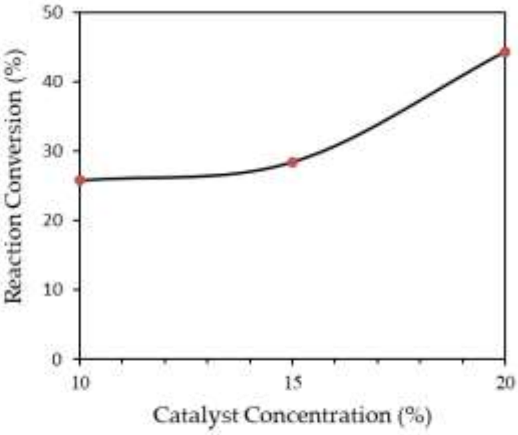

Figure 3. Effects of the lipase catalyst concentration at the reaction temperature of 40 °C and reaction time of 5 h.

3.4. Response Surface Methodology (RSM) Analysis

Response surface methodology (RSM) is the broadest employed method of experimental design for optimization. This technique applies mathematical and statistical methods for modeling and analyzing a process which involves numerous parameters. The purpose of the RSM is to optimize the response variables [29]. The factors that influence the process are entitled dependent variables. Meanwhile, the responses are named as dependent variables. Therefore, analysis using RSM is useful in designing operating conditions to achieve the targeted conversion. Many studies have shown that the most applicable tools in RSM for optimization of biodiesel production process are the Box–Behnken Design (BBD) and Central Composite Design (CCD) tools [50]. BBD is favored over CCD since it provides higher efficiency but more economical [51,52]. To evade the failure and extreme reaction variables value, BBD is limited to three levels. Hence, BBD typically has no factorial points. The BBD tool was also utilized by Sharma et al. [53] and Rokni et al. [54].

In this study, BBD was applied to obtain the best operating conditions and highest conversion for biodiesel synthesis via interesterification reaction within the ranges of the parameter values studied. The Box–Behnken Design (BBD) tool in RSM was used to evaluate the effect of the independent variables (reaction time, reaction temperature, and catalyst concentration). The experimental data of the reaction conversion compared with the prediction using BBD is exhibited in Table 5.

The parameters used to obtain the appropriate model to predict the response results must match the observed model to the experimental data. Analysis of variance (ANOVA) is accomplished using the most common types of polynomial models, namely linear, interactive (2FI), quadratic and cubic can be applied to predict the response variables of experimental data. Several parameters such as sequential *p*-value, lack of fit *p*-value, adjusted R¬2, predicted R2 and Adiq precision were used to conclude the most suitable type of model for the optimization of the conversion of biodiesel produced from CSO. In this study, optimization using RSM was initially performed using the quadratic model. The ANOVA quadratic model is presented in Table 6.

Table 5. Experimental data of the reaction conversion and the prediction using BBD.

Run	Temperature, °C	Reaction Time, h	Catalyst Concentration, % *w/w*	Reaction Conversion, %		Error, %
				Experiment	**Prediction**	
1	40	3	15	12.92	18.15	40.4
2	30	3	10	22.08	19.877	9.97
3	40	5	20	48.92	48.03	1.8
4	30	3	20	27.75	27.33	1,5
5	40	5	10	25.77	26.66	3.4
6	40	3	15	18.27	18.15	0.6
7	30	1	15	23.41	24.72	5.6
8	50	5	15	14.90	13.59	8.8
9	40	1	20	13.55	12,66	6.5
10	30	5	15	22.10	23.41	5.9
11	40	3	15	23.25	18.15	21.9
12	40	1	10	35.43	36.32	2.5
13	50	1	15	26.82	25.51	4.8
14	50	3	20	12.01	14.22	18.3
15	50	3	10	23.53	23.96	1.7

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Table 6. ANOVA of the Quadratic Model.

Source	Sum of Squares	DF	Mean Square	F-Value	*p*-Value	
Model	1007.96	9	112.00	2.13	0.2098	not significant
A-Suhu	40.83	1	40.83	0.7760	0.4187	
B-Waktu	19.45	1	19.45	0.3696	0.5698	
C-Katalis	2.61	1	2.61	0.0497	0.8325	
AB	28.14	1	28.14	0.5348	0.4974	
AC	73.90	1	73.90	1.40	0.2892	
BC	507.01	1	507.01	9.64	0.0267	

A^2	32.27	1	32.27	0.6134	0.4690	
B^2	161.73	1	161.73	3.07	0.1399	
C^2	139.90	1	139.90	2.66	0.1639	
Residual	263.06	5	52.61			
Lack of Fit	209.74	3	69.91	2.62	0.2881	not significant
Pure Error	53.32	2	26.66			
Cor Total	1271.02	14				

Based on the quadratic model, it was revealed that the *p*-value was 0.2098, which did not meet the requirement of the *p*-value < 0.05 as demonstrated in Table 6. Hence, the quadratic model was not significant for this case. To overcome this obstacle, a modified model was developed to obtain the significant model [55]. Modification of the model showed that the reduced cubic model was a significant model (Table 7).

Table 7. ANOVA of the reduced cubic model.

Source	Sum of Squares	DF	Mean Square	F-Value	*p*-Value	
Model	1197.58	10	119.76	6.52	0.0428	significant
A-Suhu	40.83	1	40.83	2.22	0.2102	
B-Waktu	165.26	1	165.26	9.00	0.0399	
C-Katalis	2.61	1	2.61	0.1423	0.7252	
AB	28.14	1	28.14	1.53	0.2834	
AC	73.90	1	73.90	4.03	0.1153	
BC	507.01	1	507.01	27.62	0.0063	
A^2	32.27	1	32.27	1.76	0.2555	
B^2	161.73	1	161.73	8.81	0.0412	
C^2	139.90	1	139.9	7.62	0.0508	
A^2B	189.62	1	189.62	10.33	0.0325	
Residual	73.44	4	18.36			
Lack of Fit	20.12	2	10.06	0.3773	0.7260	not significant
Pure Error	53.32	2	26.66			
Cor Total	1271.02	14				
Adeq Precision	9.6401					

It was found that the *p*-value of the modified cubic model was 0.0428, which met the requirement of *p*-value > 0.05. Furthermore, the value of lack-of-fit was examined. Lack-of-fit is a test that analyzes how satisfactory the full models suit with the data. Models with a significant lack-of-fit should not be applied for predictions. In this work, the lack-of-fit was not significant, which meant that the model could be employed for the prediction. The value of adeq precision is the measure of the ratio of the signal to the disturbance. The expected value of the ratio is > 4 [50]. This model provided the value of the Adeq precision of 9.6401, indicating that the model is proper. Therefore, the reduced cubic model can be used to describe the response on the reaction conversion. The empirical correlation of the reduced cubic model in the form of polynomial order for the reaction conversion in the CSO esterification is displayed in Equation (3).

$$\text{Conversion (\%)} = 18.15 - 2.26\,A + 6.43\,B - 0.5715\,C - 2.65\,AB - 4.30\,AC + 11.26\,BC - 2.96\,A^2 + 6.62\,B^2 + 6.16\,C^2 - 9.74\,A^2B \quad (3)$$

where, A is the reaction temperature (°C), B is the reaction time (min) and C is the Catalyst Concentration (%). Based on the reduced cubic model, analysis on the effects and the process variables interaction on the response variable were carried out using 3D RSM graphs. The results are presented in Figure 4.

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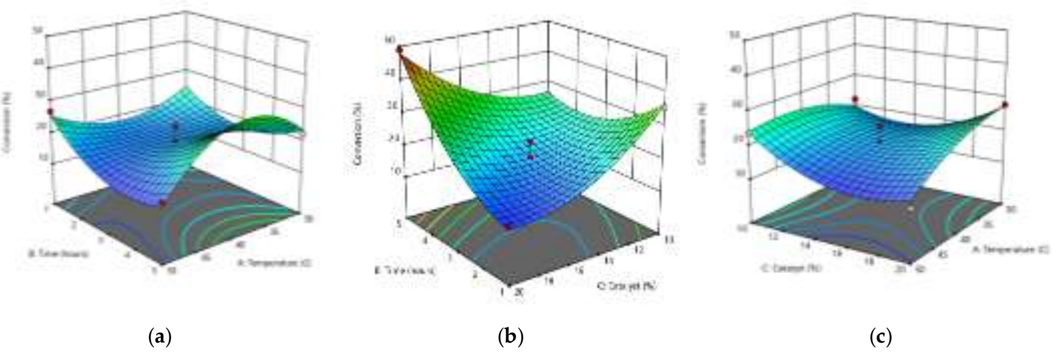

Figure 4. The 3D RSM graph representing the effects of (a) reaction time and temperature; (b) reaction time and catalyst concentration; (c) reaction temperature and catalyst concentration to the reaction conversion.

Figure 4a shows the interaction between temperature and reaction time which gives a significant change to the increase in conversion. It was found that there was an increase in conversion at the temperature of 40 °C and reaction time of 5 h. This phenomenon is in line with the investigation of Handayani et al. [55], which reported that the optimum conversion was obtained at the temperature of 45 °C and reaction time of 6 h. Figure 4b discloses that the longer the reaction time employed, the higher the conversion achieved, as the reaction time is directly proportional to the amount of product yielded. Li et al. [56] observed that the reaction conversion of *Pseudomonas cepacia* lipase was low at the reaction time of 3 h. (44%). On the contrary, it rose to 73% when the reaction time was extended to 12 h. Figure 4c bares that the lower the reaction temperature applied, the higher the conversion. This occurred because the lipase enzyme activity worked at the temperature of 30–45 °C. In the enzymatic reaction, the increase of the temperature will promote the reaction rate and accordingly the reaction conversion due to the decrease of the viscosity which enhance the solubility of oil and methanol. However, at a certain temperature, the reaction conversion declined, invoking the enzyme deactivation [57]. Meanwhile, the addition of the catalyst concentration increased the reaction conversion. This result was in line with the description of Gusniah et al. [46]. This indicated that increasing the amount of the enzyme employed will provide a higher amount of the available active sites for the reaction, leading to a higher reaction conversion.

The Derringer method is utilized to optimize the response which is characterized by the presence of a desirability function. Desirability shows how close the optimization results are to the optimum point. The desirability function (DF) is constructed on the transformation of all the acquired responses from different scales into a scale-free value. The values of DF range from 0 to 1. The value 0 means that the factors provide an undesirable response. On the other hand, the value 1 denotes the optimal performance of the evaluated factor [58]. Hence, the desirability value which is close to 1 is the expected value. However, the RSM analysis is not only to find the desirability value, which is equal to 1, but to determine the conditions that match expectations. The multiple correlation coefficient (R^2) was closed to 1. It denoted that the models have good predictive ability. Based on the RSM analysis results, the highest reaction conversion was 41.46%, attained at a temperature of 44.43 °C, a reaction time of 5 h, and a catalyst concentration of 20%, with a desirability value of 0.733 as shown in Figure 5. This finding can be stated as the local optimum within the certain parameter range studied, which is worthwhile as a basis for developing process intensification in order to obtain a higher conversion.

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"**Figure 4.** The 3D RSM graph representing the effects of (a) reaction time and temperature; (b) reaction time and catalyst concentration; (c) reaction temperature and catalyst concentration to the reaction conversion."

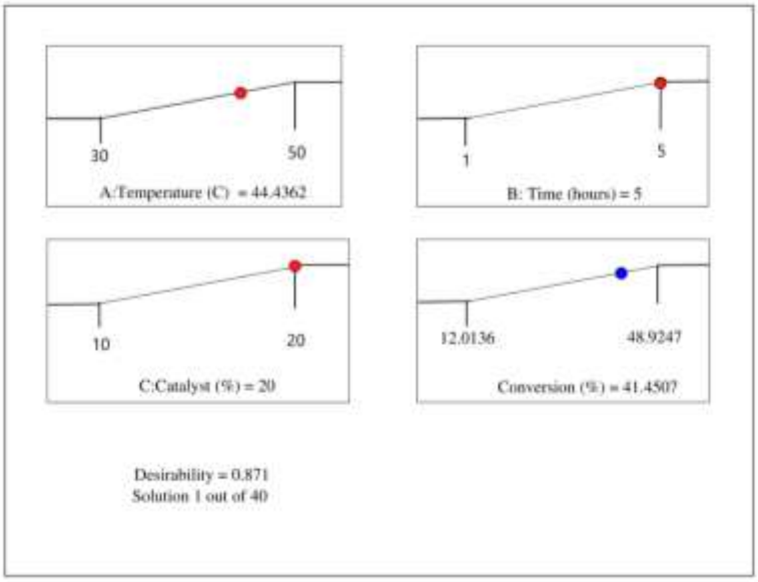

Figure 5. The highest reaction conversion based on the RSM analysis.

4. Conclusions

Based on the RSM analysis, it was revealed that the reaction time and catalyst concentration had significant effects on the conversion of the interesterification reaction of CSO with ethyl acetate in the presence of lipase catalysts for biodiesel synthesis. The results of the analysis showed that the highest reaction conversion was 41.46% at the reaction temperature, reaction time, and catalyst concentration of 44.40 °C, 5 h, and 20%, respectively, which can be stated as the local optimum. While the results are good, it is not economically feasible for industrial-scale biodiesel production and purposes in its current condition. Therefore, further process intensification is necessary to enhance the reaction conversion and biodiesel yield.

Author Contributions: Conceptualization, R.D.K. and H.V.; methodology, D.H. and F.H.; software, H.P.; validation, R.D.K., D.H. and H.P.; formal analysis, N.N. and E.D.N.A.; investigation, R.D.K., H.P., N.N. and E.D.N.A.; resources, R.D.K.; data curation, N.N. and E.D.N.A.; writing—original draft preparation, R.D.K.; writing—review and editing, R.D.K., H.P. and M.N.M.R.; visualization, N.N. and E.D.N.A.; supervision, R.D.K.; funding acquisition, R.D.K. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement:

Conflicts of Interest: The authors declare no conflict of interest

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Article

Synthesis of Biodiesel via Interesterification Reaction of *Calophyllum inophyllum* Seed Oil and Ethyl Acetate over Lipase Catalyst: Experimental and Surface Response Methodology Analysis

Ratna Dewi Kusumaningtyas [1,*], Normaliza Normaliza [1], Elva Dianis Novia Anisa [1], Haniif Prasetiawan [1], Dhoni Hartanto [1], Harumi Veny [2], Fazlena Hamzah [2] and Miradatul Najwa Muhd Rodhi [2]

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Abstract: Biodiesel is increasingly being considered as an alternative to the fossil fuel as it is renewable, nontoxic, biodegradable, and feasible for mass production. Biodiesel can be produced from various types of vegetable oils. *Calophyllum inophyllum* seed oil (CSO) is among the prospective nonedible vegetable oils considered as a raw material for biodiesel synthesis. The most common process of the biodiesel manufacturing is the transesterification of vegetable oils which results in glycerol as a by-product. Thus, product purification is necessary. In this work, an alternative route to biodiesel synthesis through interesterification reaction of vegetable oil and ethyl acetate was conducted. By replacing alcohol with ethyl acetate, triacetin was produced as a side product rather than glycerol. Triacetin can be used as a fuel additive to increase the octane number of the fuel. Therefore, triacetin separation from biodiesel products is needless. The interesterification reaction is catalyzed by an alkaline catalyst or by a lipase enzyme. In this study, biodiesel synthesis was carried out using a lipase enzyme since it is a green and sustainable catalyst. The interesterification reaction of CSO with ethyl acetate in the presence of a lipase catalyst was conducted using the molar ratio of CSO and ethyl acetate of 1:3. The reaction time, lipase catalyst concentration, and reaction temperature were varied at 1, 2, 3, 4, 5 h, 10%,15%, 20%, and 30 °C, 40 °C, 50 °C, 60 °C, respectively. The experimental results were also analyzed using response surface methodology (RSM) with the Box–Behnken design (BBD) model on Design Expert software. Data processing using RSM revealed that the highest conversion within the studied parameter range was 41.46%, obtained at a temperature reaction of 44.43 °C, a reaction time of 5 h, and a lipase catalyst concentration of 20%.

Keywords: biodiesel; *Calophyllum inophyllum* seed oil; interesterification; enzymatic; Box–Behnken design

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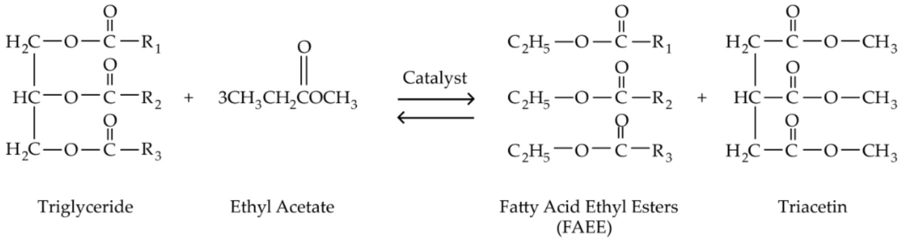
1. Introduction

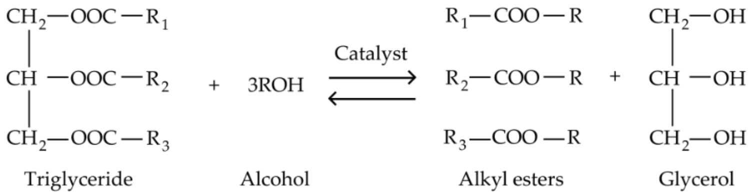
Biodiesel is an alternative fuel for diesel engines which is synthesized from vegetable oils or animal fats. Biodiesel is one of the modern bioenergies which has several advantages. It has non-toxic properties, low emission rates and no sulfur gas, just to name a few. Thus, biodiesel is a prospective alternative as an environmentally friendly diesel fuel [1]. Biodiesel is commonly derived from vegetable oil which has renewable characteristics. The main components of vegetable oils and animal fats are triglycerides, which can be converted into mono alkyl esters of long chain fatty acid or termed as fatty acid alkyl ester (biodiesel). Various types of vegetable oils can be applied as prospective raw materials for biodiesel preparation [2]. The widely used raw material for biodiesel synthesis in Indonesia is crude palm oil (CPO). CPO is abundantly available since it is produced on a large scale. However, the production of palm oil biodiesel competes with food needs. Therefore, other alternative vegetable oils are needed as raw materials for biodiesel. Nonedible oil feedstocks are favorable to ensure the sustainability biodiesel production [3,4].

There are several nonedible oils that have been widely investigated as biodiesel feedstocks, such as silk–cotton or *Ceiba pentandra* seed oil [5], jatropha oil [4,6], castor oil [7], rubber seed oil [8], karanja oil [9], mahua oil, neem oil [10], waste cooking oil [11], palm fatty acid distillate [12] and *Calophyllum inophyllum* seed oil [13]. One among the potential nonedible vegetable oils in Indonesia is *Calopyllum inophyllum* seed oil (CSO). It is also known as tamanu or nyamplung seed oil. Akram et al. [1] reported that *Calopyllum inophyllum* (tamanu) seed has an oil content of 65–75%, which is higher than other nonedible seed oil plants, such as jatropha seed oil (27–40%), rubber seed kernel oil (40–50%), and castor oil (45–65%). Rasyid et al. [14] reported that the fatty acids composition of CSO comprises of oleic acid, palmitic acid, linoleic acid, and stearic acid. According to Adenuga et al. [13], CSO biodiesel meets the Australian, ASTM and EN standards. Therefore, CSO is suitable to be used as a feedstock in the production of biodiesel. However, CSO contains high gummy substances, especially in the form of lipoid A (the hydratable phosphatides) and lipoid B (the non-hydratable phosphatides [15,16], waxes, and other impurities [17]. The existence of gum is unfavorable since it may lower the reaction rate which in consequence reduces the biodiesel yield. At an industrial level, this condition leads to an economic disadvantage. Besides, the presence of phosphorus compounds can generate the carbon particle deposition in diesel engine which causes plugging in the engine filter, line, and injectors, as well as reducing engine performance [17,18]. Thus, a degumming process is essential as the pretreatment step of biodiesel feedstocks with a high phosphorus content [19].

The most general method for making biodiesel is an alkaline-catalyzed transesterification reaction by reacting vegetable oils with short-chain alcohols such as methanol or ethanol [20,21]. However, the transesterification reaction has a limitation in terms of the potential for a saponification side reaction if the oil feedstock contains high free fatty acid (FFA) 2 mgKOH/g, as described by Kusumaningtyas et al. [4] and Sebastian et al. [22]. Besides, the transesterification reaction results in glycerol as a by-product, which needs a separation process to obtain high purity biodiesel. Biodiesel purification from glycerol by-products requires a series of separation steps which brings in high operation costs. To overcome these problems, it is necessary to apply the process of producing biodiesel which is glycerol-free through an interesterification reaction [23]. In the interesterification reaction, the use of methanol or ethanol is replaced with methyl acetate or ethyl acetate. Interesterification of triglyceride yields a triacetin co-product instead of glycerol [24]. Triacetin has an advantage as a fuel additive, a good anti-knocking, and can enhance the octane number. The addition of triacetin to the fuel also offers an environmental benefit since it reduces the exhaust smoke and NO emissions to some extent. It can be stated that the existence of the triacetin co-product in biodiesel contributes to engine performance improvement [25], as the addition of triacetin increases the oxygen content of the fuel [26]. Thus, it is unnecessary to separate triacetin from biodiesel products. A comparison between transesterification and interesterification reaction schemes are presented in Equations (1) and (2), respectively.



$$\text{Triglyceride} + 3CH_3CH_2COCH_3 \underset{}{\xrightarrow{\text{Catalyst}}} 3\,C_2H_5-O-\overset{O}{\overset{\|}{C}}-R_{1,2,3} + \text{Triacetin} \quad (1)$$



(2)

To date, the development of green energy through environmentally friendly processes has become a priority. One alternative towards a greener process is reducing the use of chemical catalysts and switching to enzymatic catalysts (biocatalysts). The enzyme-catalyzed biodiesel synthesis shows superiority compared to the chemically catalyzed one, mainly in terms of the lower energy requirement and natural conservation aspect. Lipase is the most common enzyme employed for biodiesel synthesis. There are two principal classes of lipase, free enzymes and immobilized enzymes. Free lipase enzymes are cheaper and simpler, thus it is feasible for large-scale applications [27]. Besides that, the use of lipase enzymes is very promising to overcome the disadvantages of alkaline catalysts related to the occurrence of the undesired saponification reaction when high FFA oil used as raw material. Lipase enzyme catalysts can be applied with high FFA feedstocks without any necessity of FFA removal as a pretreatment step. However, lipase biocatalysts are easily deactivated in an alcoholic environment since short-chain alcohols often cause irreversible loss of enzyme activity [28]. Therefore, the non-alcoholic interesterification route is preferable in order to maintain high biocatalyst activity and stability during the reaction. In the interesterification reaction, alcohol can be replaced by methyl acetate or ethyl acetate, which is harmless for the lipase catalyst [29].

In this work, biodiesel was synthesized through lipase-catalyzed interesterification of *Calopyllum inophyllum* seed oil (CSO) with ethyl acetate. The lipase enzyme used was the liquid-free lipase from Novozyme. The effects of the main parameters on the reaction conversion were studied experimentally. Analysis using the response surface methodology was also carried out to investigate the best reaction conversion obtained within the values range of the studied independent variables. Process optimization was also conducted using the response surface methodology, a collection of mathematical and statistical calculation techniques based on the compatibility of the empirical model with the experimental data obtained. Generally, RSM is useful for the modeling and analysis of problems that influences the response variables with the purpose of optimizing the response. RSM is also beneficial when determining the operating conditions to achieve the desired conversion [30]. There are several experimental design models used in RSM, such as the Box–Behnken Design (BBD), control composite design (CCD) and Doehlert Design. The BBD has been broadly applied for biodiesel production optimization since it is effective, requires the smallest number of trials than the other designs [31]. In this investigation, analysis using BBD in RSM was performed to determine the best reaction operation condition which resulted in the highest reaction conversion of the interesterification reaction of CSO in the presence of lipase enzyme catalyst within the ranges of the parameter values studied.

2. Materials and Methods

2.1. Materials

The materials used were: *Calophyllum inophyllum* seed oil (CSO) from UMKM Samtamanu Cilacap, Indonesia, ethyl acetate p.a (Merck), liquid lipase catalyst (Eversa Transform 2.0 from Novozyme), phosphoric acid p.a (Merck), KOH p.a (Merck), oxalic acid p.a (Merck), ethanol p.a (Merck), and aquadest from Indrasari chemicals store, Semarang, Indonesia. The catalyst used was liquid (free form) lipase Eversa Transform 2.0 (ET 2.0) from Novozyme, which is made from genetically modifed Aspergillus oryzae microorganism [32]. Commonly, lipase are active in broad range of temperature from 20–60 °C [33,34] and the ET 2.0 was reported to work satisfactorily up to the reaction temperature of 60 °C [35].

2.2. Method

2.2.1. Degumming

Crude CSO as much as 100 mL was put into a 250 mL flat bottom flask. The oil was then heated using hot plate equipped with a magnetic stirrer at a speed of 600 rpm until the temperature reached 70 °C. Phosphoric acid was subsequently added to the hot oil with the amount of 0.3% (*w/w*) of CSO and heated for 25 min. The oil was cooled at room temperature and put into a 500 mL separatory funnel. Distilled water at a temperature of 40 °C was thereafter added with a volume of 5% of the CSO volume. Subsequent to the degumming process, the mixture was settled in the separatory funnel for 24 h to attain the completion of the separation between the CSO and the residue. After the 24 h decantation process, the two layers were appeared. The top layer of brownish yellow color was the refined CSO, while the dark brown bottom layer was gum, impurities, and water which should be separated. Let the mixture in the separatory funnel for to attain the completion of the separation between the oil and the residue. The degummed CSO was then separated from the residue and was heated in the oven with a temperature of 105 °C to reduce the water content [30]. CSO was then analyzed to determine its physical properties (density, viscosity, and acid number).

2.2.2. Interesterification Reaction

After the degumming process, CSO was used as the raw material for biodiesel synthesis via interesterification reaction. CSO as much as 100 mL was introduced into a 250 mL flat bottom three neck flask and added with 10% (*w/w*) liquid lipase. The mixture was heated using a hot plate equipped with a magnetic stirrer with the stirring speed of 600 rpm until it reached the desired temperature. On the other hand, ethyl acetate was warmed up separately up to the similar temperature. When the reactants attained the specified temperature, ethyl acetate was subsequently poured into the reactor and mixed with the CSO and lipase. This incident was recorded as the reaction time of zero (t = 0 h). The ratio of CSO to ethyl acetate was fixed at 1:3. The stoichiometric ratio of the reactants was employed to investigate the optimum reaction conversion that can be obtained without any excess reactant (ethyl acetate) as it was conducted by Manurung et al. [36]. The reaction temperature was varied at 30 °C, 40 °C, 50 °C and 60 °C.The reaction temperature was varied at 30 °C, 40 °C, 50 °C and 60 °C. After the specified temperature was reached, the ethyl acetate with the similar temperature was added with the ratio of CSO to ethyl acetate of 1:3 (stoichiometric ratio). The reaction time was 1–5 h. Sample was taken periodically every 1 h of the reaction time. Prior to the analysis, sample was centrifuged with the centrifugation speed of 40,000 rpm for 15 min to separate between biodiesel product and the catalyst residue. The sample analysis was carried out using Gas Chromatography-Mass Spectroscopy (GC-MS). The independent variables for the experimental work is shown in Table 1.

Table 1. Independent Variables.

Variable	Values
Reaction Time	1, 2, 3, 4, and 5 h
Reaction Temperature	30 °C, 40 °C, 50 °C, and 60 °C
Catalyst Concentration	10%, 15%, and 20%

2.2.3. Gas Chromatography-Mass Spectroscopy (GC-MS)

Analysis of the fatty acid composition of the biodiesel was determined using a Gas Chromatography-Mass Spectrometer (GC-MS QP2010 SE) with a column flow rate of 1.20 mL/min, an oven column temperature of 65 °C, a pressure of 74.5 kPa, and an injecttion temperature of 250 °C with split injection mode. The internal standard used was heptadecanoic acid, methyl ester.

2.2.4. Response Surface Methodology Analysis

The GC-MS data was utilized to determine the reaction conversion. The results were subsequently analyzed using response surface methodology (RSM) with the Box–Behnken Design (BBD) model on Design Expert software to determine the optimum operation condition. The BBD is a vigorous and extensively applied model for biodiesel synthesis optimization and parameter analysis [31]. The levels of the tested parameters for the BBD experiment are presented in Table 2.

Table 2. Levels of Tested Parameters for Box–Behnken Design (BBD).

Independent Factor	**Units**	**Symbol**	**Level**	
			Low	**High**
Temperature	°C	A	30	50
Reaction Time	hours	B	1	5
Catalyst Concentration	%	C	10	20

3. Results and Discussion

3.1. Effects of the Degumming Process on the CSO Properties

Naturally, crude CSO contains phospholipids, sterols, free fatty acids, waxes, oil-soluble pigments and hydrocarbons. Degumming is the initial and essential refining process of crude vegetable oil to remove phospholipids and gums [37]. To date, there are various methods that can be employed to degum vegetable oil, explicitly water, acid, enzymatic, and membrane degumming techniques. Water degumming and acid degumming are the most used methods for vegetable degumming at an industrial scale [38]. Those methods are simple, easy, and low cost. However, water degumming is less effective compared to the acid method. The gum is comprised of hydratable (HP) and non-hydratable phosphatides (NHP). HP can be eliminated using water. In contrast, NHP cannot be eradicated via water degumming [15,16]. Thus, the acid degumming method is more advantageous in that it can remove both the HP and NHP. However, water degumming is less effective compared to acid degumming. Thus, the acid degumming method is more advantageous. The most used acids for the degumming process are phosphoric or citric acid, with a suggested concentration between 0.05–2% *w/w* oil [39]. In this work, 0.3% phosphoric acid was applied [23,40]. After the degumming process, the black color of the CSO turns reddish-yellow due to the loss of the phospholipids compound in crude CSO. Phospholipids can form dark colors in vegetable oil as a result of the autoxidation process of these compounds during storage. The condensation reaction between the amino groups in phospholipids with aldehydes will yield melanophosphatides compounds which give a dark color to the oil [41]. Thus, removal of phospholipids in CSO will reduce the occurrence of the abovementioned reaction and diminish the color intensity.

The degumming process can improve the properties of CSO. In this work, the density, viscosity, and acid number of crude and degummed CSO were tested experimentally. The effects of the degumming process on the physicochemical properties of CSO is indicated in Table 3. It was obvious that the density and viscosity of the CSO were slightly decreasing after the degumming process due to the removal of gum and other impurities. The effect of the degumming process on the CSO properties is indicated in Table 2. It was revealed that the density and viscosity of the CSO slightly decreased after the degumming process. It occurred with the removal of gum and other impurities. Besides, degumming also leads to a lessening of the acid number of the oil which is attributable to the decrease of the acid number and the existence of free fatty acids (FFA) in the oil during the degumming process. This phenomenon is in line with the data reported by Adekunle et al. [42]. The fatty acid composition of the CSO after undergoing the degumming process is presented in the Table 4, based on the interpretation of the chromatogram shown in Figure 1.

Table 3. Effects of Degumming Process on the Properties of CSO.

CSO Properties	Before Degumming	After Degumming
Density (kg/m^3)	941	937
Viscosity (mm^2/s)	63.42	59.73
Acid Number (mg KOH/g CSO)	64.62	48.24
Free Fatty Acid Content (%)	32.47	24.25

Table 4. Fatty Acid Composition of the CSO after Degumming Process.

Fatty Acid	Molecular Weight (g/mol)	Area (%)
Palmitic Acid	256.22	7.82
Linoleic Acid	280.45	16.82
Oleic Acid	282.52	26.62
Stearic Acid	284.47	8.86
Arachidic Acid	312.54	0.31

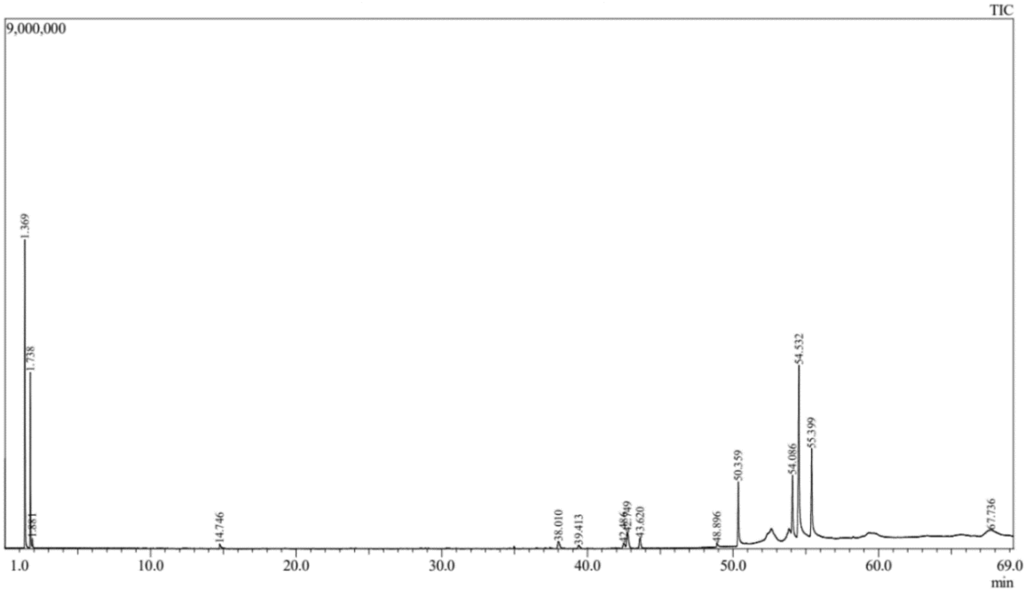


Figure 1. Chromatogram of the CSO after Degumming Process.

3.2. *Effects of the Temperature on the Interesterification Reaction Conversion*

After the degumming process, CSO directly underwent the interesterification process with ethyl acetate in the presence of the lipase catalyst. As depicted in Table 2, the crude and degummed CSO contained high FFA of 32.47% and 24.25%, respectively, which were over 2 mgKOH/g. However, the enzymatic reaction is generally insensitive to the existence of the FFA impurities in the oil feedstock. The possibility of the undesired saponification reaction can be neglected. Therefore, a specific pre-treatment for reducing the FFA content of CSO was considered unnecessary since the FFA presentation in the oil does not affect the enzymatic catalyzed reaction [22,43].

The influences of the reaction time and temperature on the reaction conversion were observed at a fixed lipase catalyst concentration of 10% (*w*/*w*). The result is exhibited in Figure 2. Basically, lipases are active in a temperature range of 20–60 °C [33,34] and the ET 2.0 lipase can work up to 60°C [35]. Moreover, the reaction time is often studied for up to 12 h, but the significant reaction rate is in the range of 0–5 h [44]. In this work, it was disclosed that the highest reaction conversion was 54.99%, obtained at a reaction temperature of 40 °C and a reaction time of 3 h. Commonly, the reaction conversion is enhanced with the increasing of the reaction temperature and reaction time, since at the higher temperature the viscosity of the liquid decreases, causing the higher solubility of reactants, mass diffusion, rate of reaction, and conversion [45]. However, in this work, the reaction conversion decreased when the reaction was performed at 50 °C for 5 h. This trend was in accordance with the results recorded by Gusniah et al. [46], where it was reported that the lipase enzyme catalyst activity in the transesterification reaction of waste cooking oil reached an optimum performance at 40 °C and it declined to some extent when the temperature rose to 50 °C, which happened when the lipase catalyst began to denature, causing damage of the active part of the enzyme at the higher temperature. Beyond the optimum temperature, the lipase catalyst becomes unstable and misplaces its tertiary structure, which causes the shortfall in its activity [45]. It thus leads to the decline of the reaction rate and conversion. Generally, the lipase enzyme reached the highest activity at the temperature of 40–45 °C as stated by Murtius et al. [47], Ayinla et al. [48], and Yazid [49].

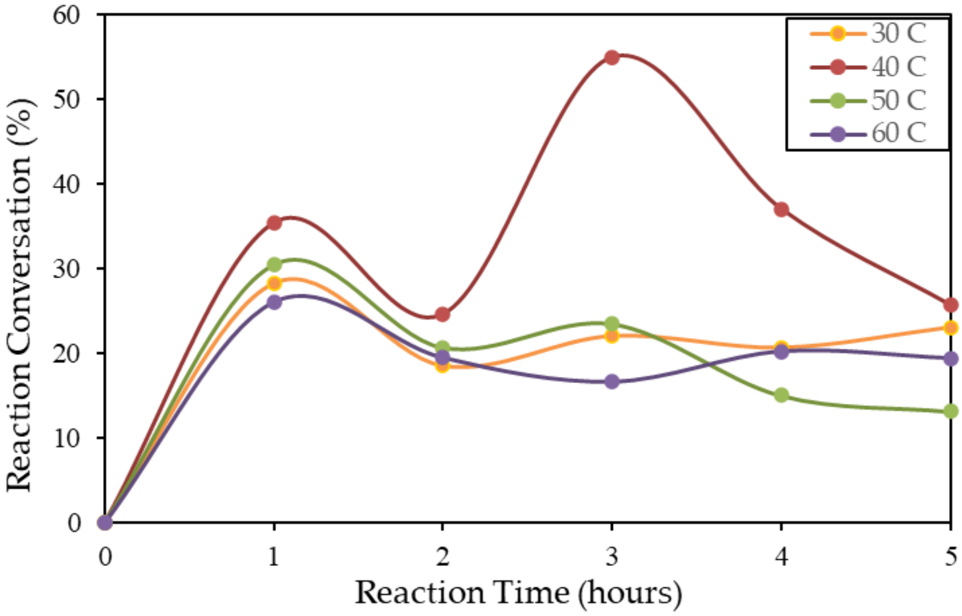


Figure 2. Effects of the reaction time and temperature on the reaction conversion at a fixed lipase catalyst concentration of 10% (*w/w*).

3.3. Effects of the Catalyst Concentration E on the Interesterification Reaction Conversion

Investigation on the influence of the lipase catalyst concentration was carried out at the molar ratio of oil to ethyl acetate of 1:3, a reaction temperature of 40 °C and a reaction time of 5 h. The lipase concentrations studied were 10%, 15%, and 20%. Figure 3 has shown that the optimum catalyst concentration was 15%, resulting in the reaction conversion of 28.445%. This result was in agreement with the results obtained by Gusniah et al. [46] which indicated that the highest lipase catalyst loading was 15%. However, the reaction conversion in this work was still low compared to those obtained in other research as the reaction was conducted with the low molar ratio of oil and ethyl acetate (1:3) and a short reaction time (5 h). By comparison, Sun et al. [44] achieved a 94.2% yield for the transesterification of semen Abutili seed oil in the presence of a similar lipase catalyst but at an excess molar ratio of oil and alcohol (1:7) and a longer reaction time (11 h).

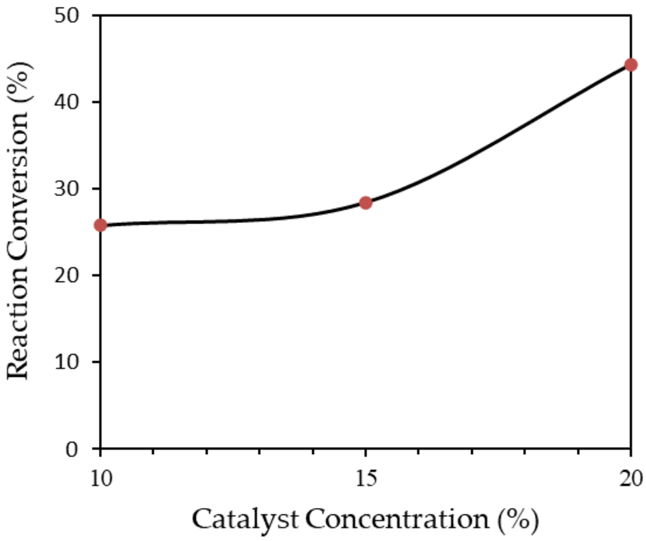


Figure 3. Effects of the lipase catalyst concentration at the reaction temperature of 40 °C and reaction time of 5 h.

Subhedar and Gogate [29] carried out the interesterification reaction of waste cooking oil and methyl acetate using immobilized lipase obtained from *Thermomyces lanuginosus* (Lipozyme TLIM) as a catalyst. The optimum reaction was demonstrated at the oil: methyl acetate molar ratio of 1:12, enzyme concentration of 6% (*w/v*), reaction temperature of 40 °C and reaction time of 24 h. Biodiesel yield was 90.1% under these operation conditions, which was higher than the results obtained in this work. A higher reaction conversion was obtained as the reaction was conducted on an excessive molar ratio and an extensively longer reaction time (24 h). Besides, this reaction employed an immobilized lipase, in which the lipase was attached on the carrier substrate, which led to higher product purity. Thus, it can be deduced that the lipase-catalyzed reaction in this work resulted in a lower conversion due to a shorter reaction time and low molar ratio of the reactants, which did not exceed the stoichiometric ratio. Besides that, utilization of the liquid lipase can reduce the apparent concentration of biodiesel yield since it can act as an impurity in the final biodiesel product.

3.4. Response Surface Methodology (RSM) Analysis

Response surface methodology (RSM) is the broadest employed method of experimental design for optimization. This technique applies mathematical and statistical methods for modeling and analyzing a process which involves numerous parameters. The purpose of the RSM is to optimize the response variables [29]. The factors that influence the process are entitled dependent variables. Meanwhile, the responses are named as dependent variables. Therefore, analysis using RSM is useful in designing operating conditions to achieve the targeted conversion. Many studies have shown that the most applicable tools in RSM for optimization of biodiesel production process are the Box–Behnken Design (BBD) and Central Composite Design (CCD) tools [50]. BBD is favored over CCD since it provides higher efficiency but more economical [51,52]. To evade the failure and extreme reaction variables value, BBD is limited to three levels. Hence, BBD typically has no factorial points. The BBD tool was also utilized by Sharma et al. [53] and Rokni et al. [54].

In this study, BBD was applied to obtain the best operating conditions and highest conversion for biodiesel synthesis via interesterification reaction within the ranges of the parameter values studied. The Box–Behnken Design (BBD) tool in RSM was used to evaluate the effect of the independent variables (reaction time, reaction temperature, and catalyst concentration). The experimental data of the reaction conversion compared with the prediction using BBD is exhibited in Table 5.

The parameters used to obtain the appropriate model to predict the response results must match the observed model to the experimental data. Analysis of variance (ANOVA) is accomplished using the most common types of polynomial models, namely linear, interactive (2FI), quadratic and cubic can be applied to predict the response variables of experimental data. Several parameters such as sequential *p*-value, lack of fit *p*-value, adjusted R¬2, predicted R2 and Adiq precision were used to conclude the most suitable type of model for the optimization of the conversion of biodiesel produced from CSO. In this study, optimization using RSM was initially performed using the quadratic model. The ANOVA quadratic model is presented in Table 6.

Table 5. Experimental data of the reaction conversion and the prediction using BBD.

Run	**Temperature, °C**	**Reaction Time, h**	**Catalyst Concentration, % *w*/*w***	**Reaction Conversion, %**		**Error, %**
				Experiment	**Prediction**	
1	40	3	15	12.92	18.15	40.4
2	30	3	10	22.08	19.877	9.97
3	40	5	20	48.92	48.03	1.8
4	30	3	20	27.75	27.33	1,5
5	40	5	10	25.77	26.66	3.4
6	40	3	15	18.27	18.15	0.6
7	30	1	15	23.41	24.72	5.6
8	50	5	15	14.90	13.59	8.8
9	40	1	20	13.55	12,66	6.5
10	30	5	15	22.10	23.41	5.9
11	40	3	15	23.25	18.15	21.9
12	40	1	10	35.43	36.32	2.5
13	50	1	15	26.82	25.51	4.8
14	50	3	20	12.01	14.22	18.3
15	50	3	10	23.53	23.96	1.7

Table 6. ANOVA of the Quadratic Model.

Source	**Sum of Squares**	**DF**	**Mean Square**	**F-Value**	***p*-Value**	
Model	1007.96	9	112.00	2.13	0.2098	not significant
A-Suhu	40.83	1	40.83	0.7760	0.4187	
B-Waktu	19.45	1	19.45	0.3696	0.5698	
C-Katalis	2.61	1	2.61	0.0497	0.8325	
AB	28.14	1	28.14	0.5348	0.4974	
AC	73.90	1	73.90	1.40	0.2892	
BC	507.01	1	507.01	9.64	0.0267	
A^2	32.27	1	32.27	0.6134	0.4690	
B^2	161.73	1	161.73	3.07	0.1399	
C^2	139.90	1	139.90	2.66	0.1639	
Residual	263.06	5	52.61			
Lack of Fit	209.74	3	69.91	2.62	0.2881	not significant
Pure Error	53.32	2	26.66			
Cor Total	1271.02	14				

Based on the quadratic model, it was revealed that the *p*-value was 0.2098, which did not meet the requirement of the p-value < 0.05 as demonstrated in Table 6. Hence, the quadratic model was not significant for this case. To overcome this obstacle, a modified model was developed to obtain the significant model [55]. Modification of the model showed that the reduced cubic model was a significant model (Table 7).

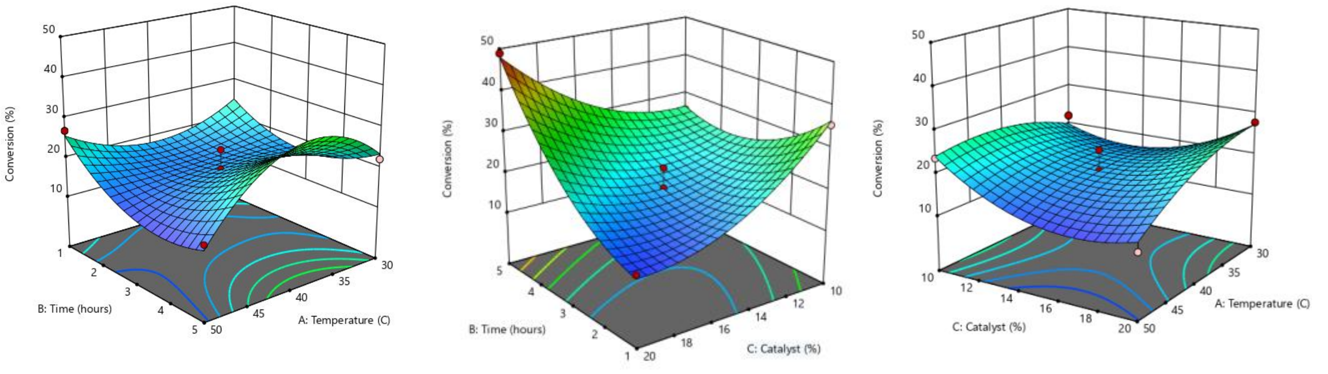
Table 7. ANOVA of the reduced cubic model.

Source	Sum of Squares	DF	Mean Square	F-Value	*p*-Value	
Model	1197.58	10	119.76	6.52	0.0428	significant
A-Suhu	40.83	1	40.83	2.22	0.2102	
B-Waktu	165.26	1	165.26	9.00	0.0399	
C-Katalis	2.61	1	2.61	0.1423	0.7252	
AB	28.14	1	28.14	1.53	0.2834	
AC	73.90	1	73.90	4.03	0.1153	
BC	507.01	1	507.01	27.62	0.0063	
A^2	32.27	1	32.27	1.76	0.2555	
B^2	161.73	1	161.73	8.81	0.0412	
C^2	139.90	1	139.9	7.62	0.0508	
A^2B	189.62	1	189.62	10.33	0.0325	
Residual	73.44	4	18.36			
Lack of Fit	20.12	2	10.06	0.3773	0.7260	not significant
Pure Error	53.32	2	26.66			
Cor Total	1271.02	14				
Adeq Precision	9.6401					

It was found that the *p*-value of the modified cubic model was 0.0428, which met the requirement of *p*-value > 0.05. Furthermore, the value of lack-of-fit was examined. Lack-of-fit is a test that analyzes how satisfactory the full models suit with the data. Models with a significant lack-of-fit should not be applied for predictions. In this work, the lack-of-fit was not significant, which meant that the model could be employed for the prediction. The value of adeq precision is the measure of the ratio of the signal to the disturbance. The expected value of the ratio is > 4 [50]. This model provided the value of the Adeq precision of 9.6401, indicating that the model is proper. Therefore, the reduced cubic model can be used to describe the response on the reaction conversion. The empirical correlation of the reduced cubic model in the form of polynomial order for the reaction conversion in the CSO esterification is displayed in Equation (3).

$$\text{Conversion (\%)} = 18.15 - 2.26\,A + 6.43\,B - 0.5715\,C - 2.65\,AB - 4.30\,AC + 11.26\,BC - 2.96\,A2 + 6.62\,B2 + 6.16\,C2 - 9.74\,A2B \tag{3}$$

where, A is the reaction temperature (°C), B is the reaction time (min) and C is the Catalyst Concentration (%). Based on the reduced cubic model, analysis on the effects and the process variables interaction on the response variable were carried out using 3D RSM graphs. The results are presented in Figure 4.



(**a**) (**b**) (**c**)

Figure 4. The 3D RSM graph representing the effects of (**a**) reaction time and temperature; (**b**) reaction time and catalyst concentration; (**c**) reaction temperature and catalyst concentration to the reaction conversion.

Figure 4a shows the interaction between temperature and reaction time which gives a significant change to the increase in conversion. It was found that there was an increase in conversion at the temperature of 40 °C and reaction time of 5 h. This phenomenon is in line with the investigation of Handayani et al. [55], which reported that the optimum conversion was obtained at the temperature of 45 °C and reaction time of 6 h. Figure 4b discloses that the longer the reaction time employed, the higher the conversion achieved, as the reaction time is directly proportional to the amount of product yielded. Li et al. [56] observed that the reaction conversion of *Pseudomonas cepacia* lipase was low at the reaction time of 3 h. (44%). On the contrary, it rose to 73% when the reaction time was extended to 12 h. Figure 4c bares that the lower the reaction temperature applied, the higher the conversion. This occurred because the lipase enzyme activity worked at the temperature of 30–45 °C. In the enzymatic reaction, the increase of the temperature will promote the reaction rate and accordingly the reaction conversion due to the decrease of the viscosity which enhance the solubility of oil and methanol. However, at a certain temperature, the reaction conversion declined, invoking the enzyme deactivation [57]. Meanwhile, the addition of the catalyst concentration increased the reaction conversion. This result was in line with the description of Gusniah et al. [46]. This indicated that increasing the amount of the enzyme employed will provide a higher amount of the available active sites for the reaction, leading to a higher reaction conversion.

The Derringer method is utilized to optimize the response which is characterized by the presence of a desirability function. Desirability shows how close the optimization results are to the optimum point. The desirability function (DF) is constructed on the transformation of all the acquired responses from different scales into a scale-free value. The values of DF range from 0 to 1. The value 0 means that the factors provide an undesirable response. On the other hand, the value 1 denotes the optimal performance of the evaluated factor [58]. Hence, the desirability value which is close to 1 is the expected value. However, the RSM analysis is not only to find the desirability value, which is equal to 1, but to determine the conditions that match expectations. The multiple correlation coefficient (R^2) was closed to 1. It denoted that the models have good predictive ability. Based on the RSM analysis results, the highest reaction conversion was 41.46%, attained at a temperature of 44.43 °C, a reaction time of 5 h, and a catalyst concentration of 20%, with a desirability value of 0.733 as shown in Figure 5. This finding can be stated as the local optimum within the certain parameter range studied, which is worthwhile as a basis for developing process intensification in order to obtain a higher conversion.

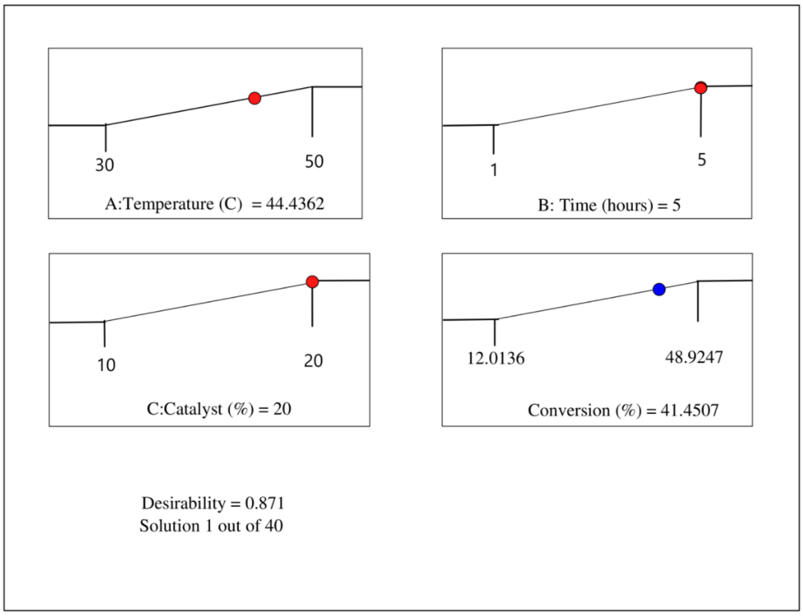


Figure 5. The highest reaction conversion based on the RSM analysis.

4. Conclusions

Based on the RSM analysis, it was revealed that the reaction time and catalyst concentration had significant effects on the conversion of the interesterification reaction of CSO with ethyl acetate in the presence of lipase catalysts for biodiesel synthesis. The results of the analysis showed that the highest reaction conversion was 41.46% at the reaction temperature, reaction time, and catalyst concentration of 44.40 °C, 5 h, and 20%, respectively, which can be stated as the local optimum. While the results are good, it is not economically feasible for industrial-scale biodiesel production and purposes in its current condition. Therefore, further process intensification is necessary to enhance the reaction conversion and biodiesel yield.

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Abstract	Biodiesel is increasingly being considered as an alternative to the fossil fuel as it is renewable, nontoxic, biodegradable, and feasible for mass production. Biodiesel can be produced from various types of vegetable oils. *Calophyllum inophyllum* seed oil (CSO) is among the prospective nonedible vegetable oils considered as a raw material for biodiesel synthesis. The most common process of the biodiesel manufacturing is the transesterification of vegetable oils which results in glycerol as a by-product. Thus, product purification is necessary. In this work, an alternative route to biodiesel synthesis through interesterification reaction of vegetable oil and ethyl acetate was conducted. By replacing alcohol with ethyl acetate, triacetin was produced as a side product rather than glycerol. Triacetin can be used as a fuel additive to increase the octane number of the fuel. Therefore, triacetin separation from biodiesel products is needless. The interesterification reaction is catalyzed by an alkaline catalyst or by a lipase enzyme. In this study, biodiesel synthesis was carried out using a lipase enzyme since it is a green and sustainable catalyst. The interesterification reaction of CSO with ethyl acetate in the presence of a lipase catalyst was conducted using the molar ratio of CSO and ethyl acetate of 1:3.

The reaction time, lipase catalyst concentration, and reaction temperature were varied at 1, 2, 3, 4, 5 h, 10%,15%, 20%, and 30 °C, 40 °C, 50 °C, 60 °C, respectively. The experimental results were also analyzed using response surface methodology (RSM) with the Box–Behnken design (BBD) model on Design Expert software. Data processing using RSM revealed that the highest conversion within the studied parameter range was 41.46%, obtained at a temperature reaction of 44.43 °C, a reaction time of 5 h, and a lipase catalyst concentration of 20%.

Keywords biodiesel; *Calophyllum inophyllum* seed oil; interesterification; enzymatic; Box–Behnken design

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