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Stepwise Methanolysis of Waste Cooking Oil Using Immobilized *Thermomyces lanuginose* Lipase within Ultrasonic-assisted Condition

***Nur Sabrina Abdul Manab*[1]**
***Harumi Veny* *,[1]**
***Azianna Gusniah*[1]**
***Sarina Sulaiman*[2]**
***Noorhaliza Aziz*[3]**
[1] *School of Chemical Engineering, College of Engineering, Universiti Teknologi MARA.Shah Alam, 40450, Malaysia*
[2] *Department of Biotechnology Engineering, Kulliyyah of Engineering, International Islamic University Malaysia*
[3] *School of Chemical Engineering, College of Engineering, Pasir Gudang Campus, Johor Branch, Universiti Teknologi MARA, Johor, Malaysia.*
**e-mail:* harumi2244@uitm.edu.my

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Abstract. Biodiesel or fatty acid methyl ester is a fuel derived from vegetable oil and animal fat. In this study, biodiesel is produced from transesterification of waste cooking oil and methanol (methanolysis), using immobilized *Thermomyces lanuginose (TLIM)* within ultrasonic-assisted conditions. The enzymatic transesterification is used due to environmental concerns and also the high yield of biodiesel. The limitations in the mass transfer rate of the product and longer reaction time have drawn attention to more efficient technology. One of them is ultrasonic-assisted to reduce reaction time and increase the catalytic activity of the enzyme. Various parameters have been examined in this study, such as reaction time, methanol to oil ratio, the effect of temperatures, and temperature dependency of immobilized *Thermomyces lanuginose (TLIM)*. The highest biodiesel yield of 69.3% was obtained after 6 hours of reaction at a temperature of 35°C and using three step-wise addition of methanol to oil ratio 3:1. Our study showed that the TLIM is sensitive to methanol to oil molar ratio above 1:1. Further research on the temperature dependence found that the TLIM activation energy in the reaction is 11.9 kcal/mol, which is within activation energy for an enzymatic reaction.

Keywords: Methanolysis, Transesterification, Ultrasonic assisted, *Thermomyces lanuginose*, Waste cooking Oil

INTRODUCTION

Nowadays, the sources of petroleum decrease day by day due to the increase in population worldwide. Therefore, many researchers have been tried finding a solution to overcome the problem and minimize the use of petroleum-based diesel in the world. One of the alternative ways is by producing biodiesel to replace petroleum diesel. Biodiesel is renewable, biodegradable, and it produces non-toxic gases. Biodiesel is also clean and environmentally safe compared to

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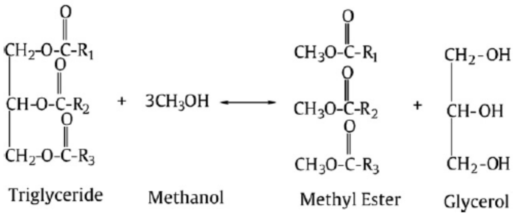
petroleum-based products (Mandolesi De Araújo et al. 2013). Biodiesel production can be derived from various sources such as vegetable oil and animal oil (Yaakob et al. 2013). Several factors need to be considered in producing biodiesel, such as the cost of raw material and food versus energy competition when using edible oil as feedstock. Thus, the alternative of producing biodiesel from recycled vegetable oil such as waste cooking oil is more economical and sustainable (Yaakob et al. 2013).

Esterification and transesterification reactions are popular methods to produce biodiesel from various feedstocks that contain free fatty acids and triglycerides (Mandolesi De Araújo et al. 2013). Transesterification is converting vegetable oil using chemicals or biocatalyst such as enzymes in the presence of alcohol, which is also known as methanolysis. The transesterification process produces biodiesel and byproduct such as glycerol. Theoretically, the stoichiometric reaction needs three moles of methanol and one mol of triglyceride. The reaction is given in Figure 1.



(1)

Fig. 1: General reaction of transesterification reaction of triglycerides with methanol (methanolysis)(Ma & Hanna, 1999).

The major drawback of chemical transesterification is downstream processing (Mandolesi De Araújo et al., 2013). Hence, transesterification using lipase enzyme as biocatalyst is an alternative process to solve the aforementioned problem. It avoids the formation of byproducts, the product is easy to recover, and the catalyst can be recycled (Vela et al. 2020). Lipase is an enzyme from living organisms, either intracellular or extracellular depending on the microorganism source (Vijayan, 2013). In this study, enzymatic transesterification is used due to the absence of a pretreatment process for raw material. Moreover, this process can be operated at low temperatures that easy to control and simple product recovery. Immobilized *Thermomyces lanuginosus* lipase (TLIM) was selected in this study as it is well known to degrade fat using its activity by controlling a plethora of vital biological processes (Bohr et al., 2019). However, the reaction time for the enzymatic process is longer compared to other methods, including acid or alkali transesterification (Carlos A., Andrés, & Fabio E., 2011).

One of the technologies to reduce process time is ultrasonic technology. Ultrasonic, also known as ultrasound, is characterized as sound with recurrence past human ear can react (Lam, Lee, & Mohamed, 2010). Ultrasonic technology is an efficient method to reduce the mass transfer rate. It also provides activation energy to start the reaction, which can minimize the energy consumption of the process. The studies from Nelson, Foglia, & Marmer (1996) showed that using a conventional transesterification with *Novozym 435* may take a reaction time of 16 hours. Thus, the use of ultrasonic is introduced to reduce reaction time, lower content enzyme, and high efficient enzyme sustainability as well as catalytic activity for enzyme-based application (Gharat & Rathod, 2013, Yan & Yan, 2017). According to a study by Ji et al (2006), a sufficient mixing with ultrasonic between immiscible liquid-liquid phase may upgrade the mass transfer efficiency and help for better mixing.

In this study, the effect of temperature dependence has also been examined. The reaction rate can be calculated using the Arrhenius equation to determine the rate constant of the process. The equation is shown below,

$$k = Ae^{Ea/_{RT}} \tag{1}$$

Where the value of Ea represents the activation energy for the reaction and the frequency factor is represented as A. The value of k is a rate constant for the reaction order, and the value of R is a gas constant at 8.3145 J/mol.K (Sreedhar & Kirti, 2016). The activation energy of TLIM in reaction will be determined and compared with the activation energy of an enzymatic reaction.

Other various parameters that may affect the biodiesel yield in methanolysis are examined, such as reaction time and the molar ratio of methanol to oil. *Thermomyces lanuginosus* lipase is also known for the hydrolysis synthesis of oil & fat. Thus, it has potential as a biocatalyst in transesterification reaction for methyl ester synthesis. In this study, the effect of methanol inhibition towards the TLIM was investigated to avoid lipase inhibition in methanolysis. The result from this study is expected may contribute to understanding the behavior of TLIM in transesterification with methanol and determining the suitable operating condition for the reaction.

MATERIALS AND METHODS

Materials

Waste cooking oil was donated by staff and students from Chemical Engineering Faculty and placed in the collection point. The enzyme was immobilized *Thermomyces lanuginose* from Sigma Aldrich, Malaysia. Methanol, n-hexane, Potassium hydroxide, Toluene, Phenolphthalein, and FAME standard of GLC-10 were from Merck Sdn Bhd, Malaysia.

Methanolysis of waste cooking

The experiment was carried out in 500 ml of a three-neck flask with a thermometer for observing the temperature. Ultrasonic bath with model S 80H from Elmasonic, Germany, was used, with a range of frequency between 50-60 Hz and power rate of 750 W and the highest allowable temperature of 70°C.

The amount of waste cooking oil was fixed at 10 g for all experiments. The weight of the immobilized *Thermomyces lanuginose* was set at 0.5 g. The parameters being studied were reaction profile, molar ratio methanol to oil temperature, and determination of activation energy. The methanol was mixed with oil and filled into three-neck flasks before adding the enzyme. After the ultrasonic irradiation, the mixture was separated using a centrifuge for 20 minutes at 1000 rpm to separate the biodiesel, glycerol, and the immobilized enzyme.

Analysis of FAME with GC-MS

Gas Chromatography-Mass Spectrometry (GC-MS) from Shimadzu was used to analyze and identify FAME (biodiesel) content. The carbowax column was used with inlet temperature adjusted at 250°C. Initially, the sample was mixed with n-hexane after the centrifugation until homogeneous. The mixture was filled into the vial for Gas Chromatography-Mass Spectrometry (GC-MS) test.

Analysis of Free Fatty Acid

This experiment was carried out to determine the free fatty acid (FFA) content in the biodiesel by titration with NaOH. The following equation calculates the percentage of FFA for palmitic acid, linoleic acid, and oleic acid.

$$\%\,FFA = \left(\frac{V \times N \times MW_{fatty\ acid}}{W \times 1000}\right) \qquad (2)$$

The percentage of FFA contain in the waste cooking oil was found below 2% (1.6-1.8%) and reduced to below 0.5% for biodiesel.

RESULTS AND DISCUSSION

Study on reaction profile

A study on the effect of reaction time or reaction profile was initially observed at a molar ratio of methanol to oil 1:1 at 45° C within the ultrasonic-assisted condition. This lower ratio was selected to avoid any inhibition by methanol towards TLIM. Figure 2 shows the reaction profile or transesterification of waste cooking oil using TLIM and ultrasonic-assisted. The reaction is completed after 2 hours where more than 30% of biodiesel yield was achieved. This reaction time is used as a basis in deciding the time for the addition of methanol in stepwise methanolysis

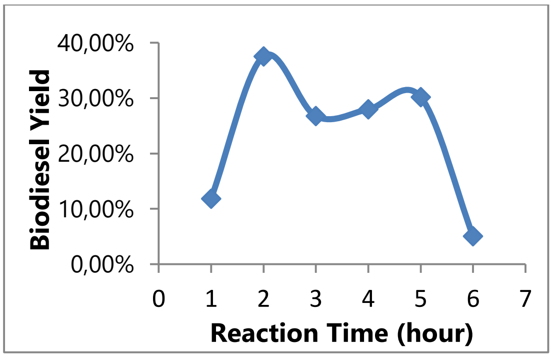


Fig. 2: Reaction Profile for transesterification of waste cooking oil for molar ratio 1:1 within ultrasonic-assisted.

Effect of Molar ratio Methanol to oil

The experiments were carried out for molar ratio methanol to oil from 1:1 to 2:1 to study the molar ratio effect and use for the three-step wise addition. Both experiments of molar ratio were set at a temperature of 45°C. The sample was collected every hour within 6 hours of reaction.

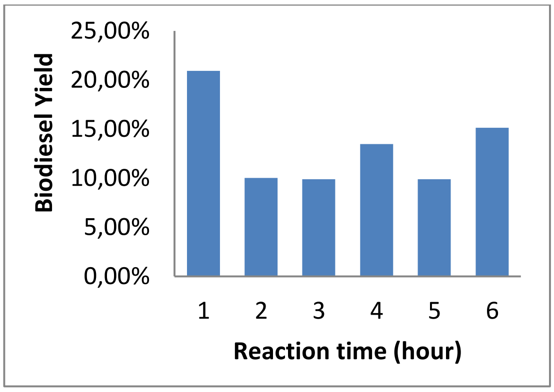


Fig. 3: Reaction profile at a molar ratio of methanol to oil 2:1

Figure 3 showed the results of methanol to oil molar ratio of 2:1. The highest conversion was found at the first hour with only a 20.93% yield compared to the theoretical yield of 66%. It is shown that the enzyme is deactivated when the molar ratio of methanol to oil reaches 2:1. Thus, the molar ratio of 1:2 methanol was not suitable for three stepwise addition due to the fast deactivation of immobilized lipase. Hence, the methanolysis needs to be pursued with three stepwise addition of methanol with the molar ratio of 1:1 for each step. This is following the options in avoiding lipase inactivated by methanol, such as methanol stepwise addition (three-step or two-step methanol addition), acyl acceptor alteration (methyl acetate, ethyl acetate), and addition of solvent (Musa, 2016)

Three stepwise methanolysis

In three stepwise methanolysis, the study was carried out at similar operating conditions of temperature 45°C. Figure 4 shows the reaction profile for the stoichiometric molar ratio of methanol to oil 3:1 with three stepwise addition of methanol. The methanol was added three times every 2 hours.

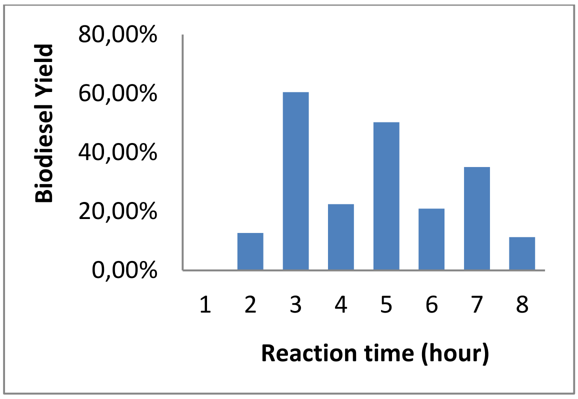


Fig. 4: Reaction profile of three-step methanolysis of methanol to molar ratio 3:1.

The highest biodiesel yield achieved was 60.45% after 3 hours of reaction. Further reaction time leads to a decrease in biodiesel yield. A comparable result was obtained by N Avlijas and F Nappi, (2009) using *Thermomyces lanuginose*. They managed to achieve a biodiesel yield of 55.5%. However, their study required a higher molar ratio of methanol to oil which was 6:1. These results show that *Thermomyces lanuginose* might not be suitable for the high molar ratio of methanol. Since the more diluted concentration of oil with methanol may cause the lower collision rate of the oil with immobilized lipase in the process due to increase turbulence flow from ultrasonic.

Effect of temperatures and temperature dependence.

According to a recent study from Šibalić et al. (2020), *Thermomyces lanuginose* lipase (TLL) is confirmed as a thermostable protein. It allows the catalysis at a broad range of temperatures (Šibalić et al. 2020). Thus, in our study, the temperatures were varied from 30° to 65°C to determine the optimum temperature for *Thermomyces lanuginose*. This experiment was carried out for 6 hours with a molar ratio of methanol to oil of 3:1. The result of biodiesel yield on temperature is shown in Figure 4.

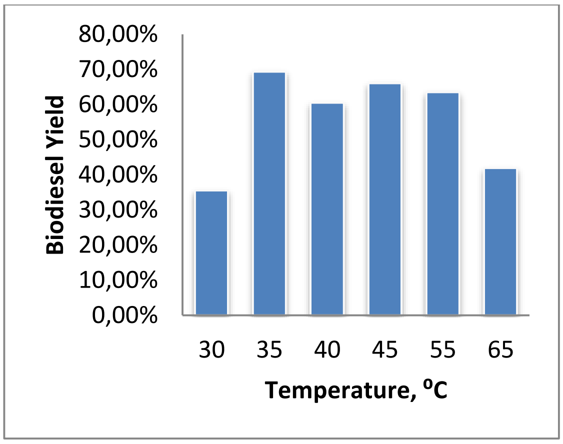


Fig. 5: Effect of different temperatures in three stepwise methanolysis of waste cooking oil.

From Figure 5, the profile graph showed that the highest biodiesel yield at a temperature of 35°C obtained 69%. The conversion of biodiesel decreases by the increasing temperature. Cancela et al. (2015) also achieved a similar result where they obtained the highest biodiesel yield at 30° C, and above this temperature, the yield was decreasing. According to Gharat and Rathod, (2013), lower viscosity leads to an increase of cavitation events and also emulsion formation rate. Nevertheless, this formation of cavitation bubble improved the mass transfer of the biodiesel that raised the biodiesel conversion (Gharat & Rathod, 2013). Different studies from Moazeni et al. (2019) obtained higher optimum temperature at 50°

C with a higher biodiesel yield of 96% within ultrasonic conditions. However, their study was using a different acyl acceptor, which is methyl acetate, with a higher molar ratio of 9:1(Moazeni, Chen, & Zhang, 2019). Thus, it showed that TLIM is more sensitive towards a higher molar ratio of methanol than methyl acetate.

The temperature dependence of immobilized T*hermomyces lanuginose* in three stepwise methanolysis within ultrasonic-assisted conditions can be observed. The experimental data were collected in various ranges from 303.15K (30° C) to 338.15K (65° C), as shown in Figure 5, to determine the rate constant. The rate reaction is calculated using the Arrhenius equation from Eq. (1). Rearranging Eq. (1), the activation energy can be determined by plotting ln k versus 1/T, as follow.

$$\ln k = -\frac{E_a}{RT} + \ln A \qquad (3)$$

Where Ea represents the activation energy for the reaction, A is the frequency factor, the k is a rate constant for the reaction order, and R is the gas constant at 8.3145 J/mol.K. The plot is shown in Figure 6.

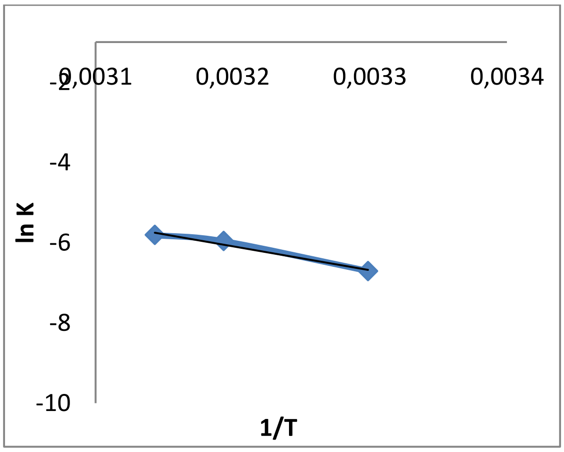


Fig. 6: The plot of ln k versus 1/T.

The activation energy was obtained by multiplying the slope with gas constant at 8.3145 J/mol.K, as calculated in equations (4) and (5).

$$-\frac{E_a}{R} = -5992.5 \qquad (4)$$

$$E_a = 49821.6\ J/mol \qquad (5)$$

The value activation energy is 49,822 J/mol.K or 11.9 kcal/mol. This activation energy is within the activation energy for an enzyme-catalyzed reaction, which is between 4-20 kcal/mol (Shuler & Kargi, 2002).

CONCLUSIONS

Methanolysis of waste cooking oil using immobilized *Thermomyces lanuginose* (TLIM) and ultrasonic-assisted was successfully investigated. In this study, the ultrasonic was proven to reduce reaction time to 2 hours. The immobilized *Thermomyces lanuginose* (TLIM) was found inhibit by methanol to oil molar ratio above 1:1. Thus, methanolysis was only possible with three stepwise additions of methanol to oil molar ratio of 1:1 for each step to achieve a stoichiometric reaction. The optimum temperature was obtained at 35^{0}C, with the highest yield obtained was 69.3%. Although the yield was lower than other studies with TLIM, the activation energy for this experiment was still within the range of activation energy for enzymes.

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