Enzymatic Transesterification Using Different Immobilized Lipases and its Biodiesel Effect on Gas Emission

Nur Fatin Syafiqah Mohamad Nor[1], Harumi Veny[1],*, Fazlena Hamzah[1],
Miradatul Najwa Muhd Rodhi[1], Ratna Dewi Kusumaningtyas[2], Haniif Prasetiawan[2],
Dhoni Hartanto[2], Sarina Sulaiman[3], Rozana Azrina Sazali[1]

[1]School of Chemical Engineering, College of Engineering, Universiti Teknologi MARA, 40450 Shah Alam Selangor, Malaysia.
[2]Chemical Engineering Department, Faculty of Engineering, Universitas Negeri Semarang Kampung Sekaran, Gunung Pati, Semarang, 50229 Indonesia.
[3]Department of Biotechnology Engineering, International Islamic University Malaysia, Kuala Lumpur, Malaysia.

Abstract

Biodiesel, a third-generation biofuel, offers several advantages over regular diesel fuel. Waste cooking oil (WCO) emerges as an ideal feedstock due to its availability and easy accessibility. In this work, biodiesel was produced from two different types of immobilized lipases: *Rhizomucor miehei* lipase (RMIM) and *Candida antarctica* lipase B (CALB). The objective of this study was to evaluate the impact of the molar ratio of WCO to methyl acetate, toward biodiesel yield and triacetin formation, from transesterification reaction when using two different immobilized lipases namely, *Rhizomucor miehei* lipase (RMIM) and *Candida antarctica* lipase B (CALB) as catalyst, as well as to assess its resulting biodiesel in a diesel engine. The enzymatic transesterification reaction was carried out with ultrasonic assistance, and the results showed that the greatest yield of 81.20% was achieved at 45 °C using CALB as a biocatalyst, with a lipase concentration of 1.8% (w/v) and an oil to methyl acetate molar ratio of 1:12 within 3 h. Triacetin, a by-product was determined their concentration for each molar ratio and analyzed using FTIR range of 500 cm^{-1} to 4000 cm^{-1}, revealing a significant absorption peak at 1238.90 cm^{-1}. Biodiesel was then blended with commercial diesel fuel in varying quantities of 7, 10, and 20% by volume (B20). The results were compared to Industrial Diesel Fuel 7% (B7) and Commercial Diesel Fuel 10% (B10). NOx and CO_2 emission drops as the percentage of diesel/biodiesel blends increases, supporting WCO as a cost-effective biodiesel feedstock with low petrol pollution.

1. Introduction

The world's population is growing quickly, and with it, the need for energy is rising. Energy is crucial for improving living standards and driving economic growth across all sectors, including agriculture, industry, transportation, commerce, and households. Fossil fuels like petroleum, coal, and natural gas have been the main sources of energy, making up about 81.1% of global energy usage [1].

The high demand for energy in industry and homes has led to environmental problems. Burning fossil fuels for energy produces harmful effects like greenhouse gas emissions, air pollution, smog, acid rain, and environmental damage. As environmental concerns grow, people are exploring alternative energy sources such as wind, hydro, solar, biomass, and biofuels. These renewable energy sources are important for both

* Corresponding Author.
  Email: harumi2244@uitm.edu.my (H. Veny)

the economy and the environment, and biodiesel is one potential solution. Biodiesel is a cleaner, more sustainable substitute for diesel made from petroleum. It produces environmentally friendly combustion emissions and poses no toxicity or cancer risks [2].

Biodiesel production involves a reaction between triglyceride and alcohol in the presence of a catalyst, with triglyceride being sourced from plants and animals. Plants used for biodiesel can be categorized into edible oils like coconut, soybean, and corn oil, and non-edible oils such as algae and jatropha [3]. However, using edible oils raises concerns about food security and environmental sustainability due to competition with food production, deforestation, and habitat destruction [4]. Animal fats can also be used, but they pose challenges due to their saturated fatty acid content [4]. To address these issues, waste cooking oil (WCO) is preferred as a primary feedstock for biodiesel production in this study. WCO is cheaper, readily available from restaurants and food processing industries, and offers similar qualities to palm oil for biodiesel production [3].

Currently, biodiesel is typically produced through acid/alkali catalyzed transesterification of an oil in the presence of inorganic basic or acid catalyst, such as KOH, NaOH and H_2SO_4, as chemical catalysts. These chemical catalysts are favored as they promote high conversion rates in relatively short reaction times [5]. However, there are drawbacks associated with chemical catalyst including high energy consumption for their activation and can make product recovery and catalyst reusability difficult. This means that the process can be energy-intensive and separating the biodiesel from the catalyst and glycerol become more challenging. Moreover, the catalysts may not be easily recoverable or reusable, leading to increased cost and environmental concerns. Thus, enzyme-catalyzed transesterification of oil is a good alternative to overcome these issues and the most greenways to produce biodiesel is by using enzyme which is lipase as bio-catalyst. This is because of its specific reaction mechanism where it is attributed to the presence of an amphiphilic peptide loop covering the enzyme's active site. This loop, being both hydrophilic and hydrophobic, allows the enzyme to interact with both water-soluble and oil-soluble molecules effectively. As a result of this unique structure, the enzyme exhibits high selectivity when catalyzing both esterification and transesterification reactions simultaneously. Immobilized lipase is used to overcome the issue of high cost of enzyme when compare to chemical catalyst. This is because, when the enzyme is in immobilized form, it become more stable and can be reused many times in reaction [6].

Some common types of immobilized lipases used in biodiesel production including those derived from microorganisms, such as *Pseudomonas cepacia, Candida antarctica, mucor miehei, Thermomyces lanuginose,* and many more [7]. According to Ycel *et al.* [7], *Mucor miehei* lipase is 1,3-specific lipases group which able to catalyze transesterification reactions efficiently and stated that *Candida antarctica* lipases are very suitable for transesterification reaction. In addition, lipase from *Candida antarctica* lipase B received more attentions due to its features such as feasibility in mild conditions, easy to handle, recover and reuse [8] and has high catalytic activity in a solvent free transesterification reaction [9]. Thus, this study aimed to produce biodiesel from WCO through enzymatic transesterification using two immobilized lipases which are *Candida antarctica* lipase B (CALB) and *Rhizomucor miehei* lipase (RMIM).

Common ways to produce biodiesel is by using methanol as an acyl acceptor [10]. Methanol is a short-alcohol that is often preferred for biodiesel production due to its affordability and low carbon content [11]. However, using methanol in excess can have negative consequences. Firstly, an excess of methanol can lead to the deactivation of the enzyme catalyst typically used in biodiesel production processes. Additionally, glycerol, which is a major by-product of the reaction between methanol and triglycerides, may inhibit the activity of the enzyme catalyst. This inhibition can result in a lower rate of biodiesel production [12]. Therefore, the application of alkyl ester, such as methyl acetate helps to overcome these problems. The reaction of methyl acetate with vegetable oils helps to solve problem of enzyme inhibition and can be an alternative for acyl acceptor in transesterification process instead of alcohol. The yield of biodiesel can be increased when using ester as an acyl acceptor by enhancing the dilution effect of lipids in oil sources. A lower concentration of lipids can reduce their negative impact on enzymatic activity, thereby improving the overall efficiency of the biodiesel production process [13]. As stated by Subhedar *et al.* [12], this route gives significant advantages towards biodiesel production even though the reaction is not well explored yet. In terms of economical aspect, while methanol typically serves as a common acyl acceptor due to its lower cost range of \$520-\$550 per ton [14], the choice to utilize methyl acetate at a slightly higher price range of \$600-\$700 per ton [15] suggests strategic considerations beyond mere affordability. Methyl acetate's selection may be driven by its compatibility with lipase enzymes, offering advantages, such as enhanced reaction kinetics, specificity, or stability, which can lead to improved process efficiency and biodiesel quality

[16]. The transesterification reaction with methyl acetate using enzyme as biocatalyst is as shown in Figure 1.

From Figure 1, it is shown that 1 mole of triglyceride theoretically required 3 moles of methyl acetate in order to produce 3 moles of fatty acid methyl ester (FAME) and 1 mole of triacetin as by-product instead of glycerol. Triacetin serves as a plasticizer and gelatinizing agent in polymers and explosives, enhancing their flexibility and stability. Additionally, it finds application as an additive in pharmaceuticals and cosmetics, where it serves various purposes, such as improving texture and consistency [17]. One notable advantage of triacetin is that it does not adversely effect on the activity of lipase enzyme [12], due to its compatibility with the active sites of lipase enzymes and its ability to undergo efficient hydrolysis.

Despite the significant advantages offered by the transesterification reaction involving triacetin and methyl acetate, there has been limited research exploring this route when employing enzymes as catalysts. Such as a study from Duraiarasan *et al.* [18], where they reported about 92.34% of biodiesel yield was obtained by using methyl acetate as an acyl acceptor with 1.5 g of enzyme, 1:12 molar ratio of oil to methyl acetate, temperature of 35 ℃ in 60 h of reaction time. Another advantage of using esters as acyl acceptor is its high yield even when dealing with crude oil. Du *et al.* [19], reported that this might be due to the increased presence of methyl acetate in the reaction medium, which enhances the dilution effect and mitigates the negative impact of lipids on enzymatic activity. Additionally, the process produces triacetin as a valuable by-product, which can be utilized in various industrial applications, thereby improving the overall economic viability of the biodiesel production process [19]. Also, according to them, methyl acetate yielded high results in both crude and refined oil, unlike methanol, which exhibited slower reaction and lower yields in crude oil compared to refined oil. Furthermore, lipase activity for methanol decreased to less than 70% after fourth cycle whereas with methyl acetate, its activity remained above 90% even after 100 cycles [19].

The primary issue frequently encountered in enzymatic transesterification reaction is the slowest reaction rate, which significantly influences the yield of biodiesel when respect to time [20]. This slow reaction rate can significantly impact the yield of biodiesel, especially concerning the time taken for the reaction to reach completion. The sluggish reaction rate primarily arises from poor mixing of the alcohol and oil phases, as these liquids are immiscible with each other. The lack of thorough mixing creates a lag time before the reaction can proceed, prolonging the overall reaction time and potentially reducing the yield of biodiesel. In order to shorten reaction time and increase the reaction rate of transesterification, ultrasonic technology is introduced. Ultrasonic-assisted has been explored in order to overcome the challenges encountered in transesterification through the enzymatic transesterification reactions. In the context of transesterification, ultrasonication offers several advantages. The ultrasound condition can eliminate the effect of mass transfer limitation between the immiscible liquid and enzyme as well as can generate bubbles cavitation which provide mechanical energy for mixing and cut the lag phase at initial phase of transesterification [21]. Several studies have been conducted on biodiesel synthesis with ultrasonic assisted transesterification using lipase as bio-catalyst. Most of studies have managed to obtain high biodiesel yield within short time. Such as a study from Adewale *et al.* [22] where they achieved 96.8% biodiesel yield from waste lard using *Candida Antarctica* lipase B in 20 min, while Tupufia *et al.* [23] reported 80% conversion in 50 hohours conventionally versus 92% in 3 h with ultrasonic assistance.
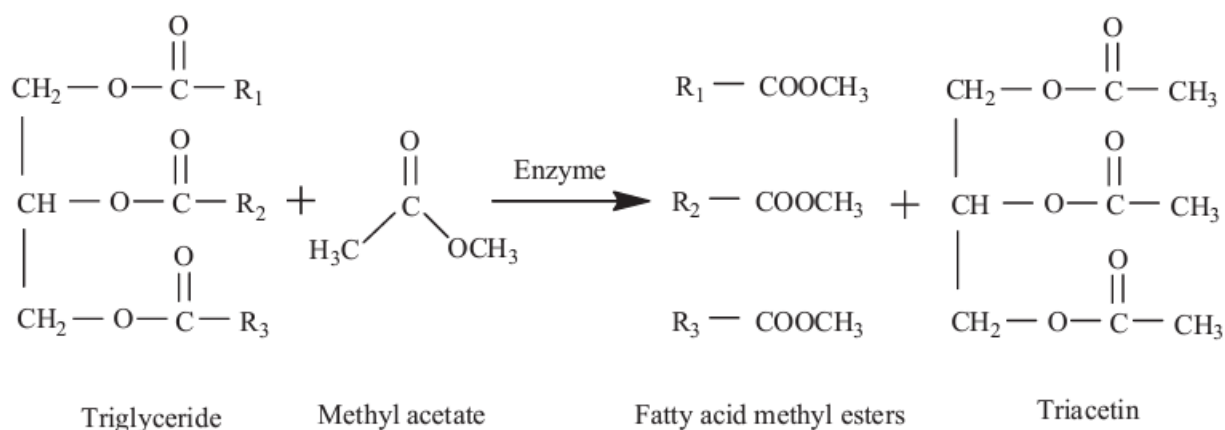

Figure 1. Transesterification reaction with methyl acetate using enzyme as biocatalyst [12].

Biodiesel production from waste cooking oil (WCO) is gaining attention as a sustainable alternative to fossil fuels due to its environmental benefits and potential for reducing dependency on finite resources. Various methods have been explored for biodiesel production, including chemical transesterification and enzymatic processes using immobilized lipase enzymes. Meanwhile enzymatic transesterification offers several advantages such as higher specificity, milder reaction conditions, and reduced waste generation compared to chemical methods, it still faces challenges related to enzyme stability, reaction efficiency, and substrate specificity. Previous research efforts have focused on optimizing reaction parameters [24,25], exploring different immobilization techniques [26,27], and investigating novel enzyme sources [28] to improve the efficiency and feasibility of enzymatic biodiesel production. However, there remains a gap in understanding the interplay between reaction conditions, enzyme characteristics, and substrate properties to achieve high yields and selectivity in biodiesel production from WCO. Other than that, lack of studies reported on the efficiency of resulting biodiesel in diesel engine. Thus, this present study aims to investigate the biodiesel production from waste cooking oil (WCO) using two types of immobilized lipase as catalysts which are CALB and RMIM, focusing on the effect of oil to methyl acetate molar ratio in an ultrasonic system and its resulting biodiesel effect on gas emissions from diesel engine, such as NOx, CO and CO_2 when blended with commercial fuel and industrial fuel. This study's outcomes could add valuable insights to the understanding of biodiesel synthesis using immobilized lipases, specifically on CALB and RMIM, as well as its impact on gas emissions from diesel engines.

2. Materials and Methods

2.1 Materials

The waste cooking oil was collected from house. *Candida antarctica* Lipase B (CALB) and *Rhizomucor miehei* lipase (RMIM), immobilized on Immobead 150, were directly purchased from Sigma Aldrich Malaysia. Both immobilized lipases were stored in a refrigerator at low temperature range of 2 °C to 8 °C. Methyl acetate and n-hexane were obtained from the laboratory while FAME Standard and mixed $C_4 - C_{24}$ were also purchased from Sigma Aldrich, Malaysia.

2.2 Methods

2.2.1 Enzymatic transesterification of WCO in ultrasonic system

The enzymatic transesterification of waste cooking oil was performed in a three-round neck flask with essential equipment such as a condenser, thermometer, and rubber stopper. The flask was positioned on an ultrasonic water bath, as depicted in Figure 2(a). A mixture of 10 mL of waste cooking oil and methyl acetate was prepared at various molar ratios at 45 °C. The molar ratios of reactants (triglyceride of waste cooking oil (WCO) and methyl acetate) were determined by applying WCO as the limiting reactant in a stoichiometric reaction. In the experiments, the reactants were prepared by converting moles into mass and volume, based on their molecular weights and densities.

The mixture of WCO and methyl acetate was added with 0.18 g (1.8% w/v) of lipase in a three-neck flask. The mixture was left for 3 hours, then centrifuged at 1000 rpm for 15 minutes to separate the enzyme and triacetin from the biodiesel. As a result, triacetin and enzymes settled at the bottom layer of the tube, as depicted in Figure 2(b). The supernatant, or upper layer resulting from centrifugation, was subsequently analyzed using GC-MS to determine the FAME content.
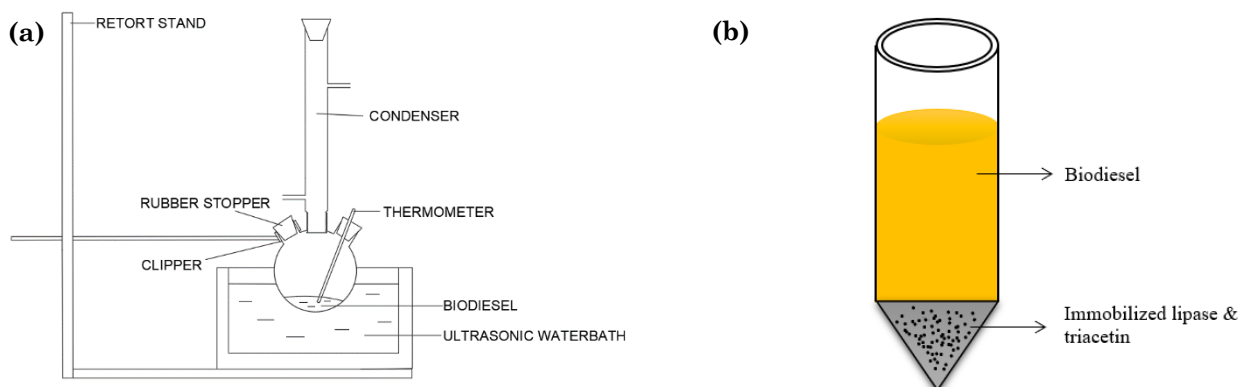

Figure 2. The arrangement of the experiment (a) for transesterification reaction and the outcome from the biodiesel (b).

2.2.2 Determination of FAME by GC-MS

Four samples were prepared with different molar ratios of WCO to methyl acetate (1:3~1:12) and diluted with n-hexane before analyzed with GC-MS. With helium as carrier gas, the SLB®-5ms GC Column was used to analyze all the samples. In GC-MS, carbowax column was installed along with FID detector. The peaks detected in the chromatogram were compared with the peak from FAME standard ($C_4 - C_{24}$). The yield of biodiesel was calculated using the following formula:

$$FAME\ yield\ (\%) = \frac{Total\ amount\ of\ FAME\ produced}{maximum\ of\ total\ FAME\ would\ be\ produced} \times 100\% \quad (1)$$

2.2.3 Engine testing performance using resulting biodiesel

The engine performance test using resulting biodiesel blend from this study, *Yanmar* Diesel Bench Dynamometer engine was used in this study and the biodiesel is mixed with industrial diesel fuel (B7) and commercial diesel fuel (B10) at different proportions of 7, 10 and 20% by volume. The diesel/biodiesel blends with their description are as shown in Table 1. A 100 mL of sample was prepared and the sample was poured into the fuel inlet and adjusted the torque for 1 Nm. The engine was switched on and at the same time the speed was adjusted by increasing it into a medium speed. The timer was started when the engine was running and stable. The data of gas analyzer and temperature of oil, exhaust and air-intake on display panel were recorded when 20 mL of sample is consumed. The procedure was repeated when the fuel was consumed to get the average results.

3. Results and Discussion

3.1. Effect of WCO to Methyl Acetate at Different Molar Ratio

In transesterification, which converts triglycerides into biodiesel, three moles of methyl acetate are required for each mole of triglycerides, indicating a stoichiometric ratio. In order to drive the reaction towards biodiesel production, methyl acetate is used in excess amount, ensuring a favorable equilibrium shift towards product formation. This excess ensures greater efficiency and higher yields by favoring the forward reaction over the reversible nature of transesterification, where the reaction can potentially revert to its starting materials.

Considering this aspect, experiments were conducted to determine the optimal molar ratio of oil for both RMIM and CALB ranging from 1:3 to 1:12, at 45 °C using 1.8% (w/v) of immobilized lipases and the obtained results is as shown in Figure 3. The experimental results obtained from effect of molar ratio in transesterification using *Rhizomucor miehei* lipase (RMIM) and *Candida antarctica* lipase B (CALB) as catalysts revealed distinct trends in biodiesel yield.

The RMIM exhibited the highest biodiesel yield of 16.6% at molar ratio of 1:3 of WCO to methyl acetate, increasing the molar ratio with this lipase would decrease the FAME yield. This phenomenon showed high amount of methyl acetate above stoichiometric molar ratio (1:3) would inhibit the activity of RMIM [13]. Conversely, CALB demonstrated significantly higher biodiesel yield, with the highest yield of 81.2% observed at a molar ratio of 1:12 of WCO to methyl acetate. This substantial increase in yield compared to other ratios suggests that excess methyl acetate favored high conversion of triglycerides into biodiesel with CALB. The surplus methyl acetate likely facilitated efficient utilization of triglycerides, leading to enhanced biodiesel yield.

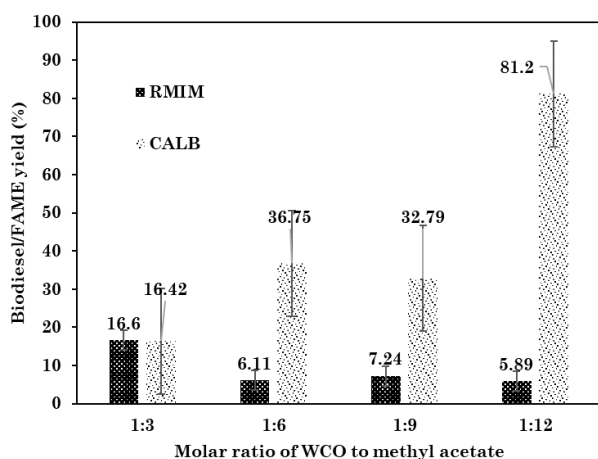On the other hand, the non-specific activity of CALB proves advantageous in biodiesel

Figure 3. Effect of waste cooking oil to methyl acetate molar ratio towards biodiesel yield.

Table 1. Diesel/biodiesel blends and its percentage blends.

Diesel/biodiesel Blends	Description
B7	Industrial fuel with 7% of biodiesel content
B10	Commercial fuel with 10% of biodiesel content
B20a	Blend of B7 with 13% of pure biodiesel
B20b	Blend of B10 with 10% of pure biodiesel

production as it maximizes the conversion by efficiently hydrolyzing all ester bonds within triglycerides, CALB ensures that all accessible fatty acid moieties participate in the production
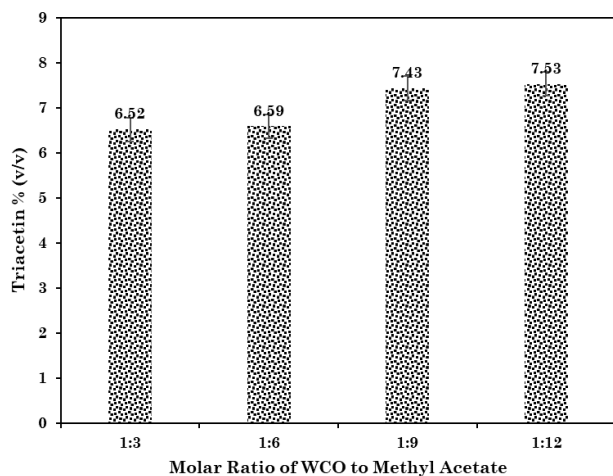

Figure 4. Triacetin concentration with different molar ratios.

Figure 5. FTIR spectra of triacetin from transesterification of waste cooking oil.

processes, resulting in higher biodiesel yield. Contrarily, RMIM's specificity towards sn-1,3 positions might lead to incomplete hydrolysis of triglycerides [29], leaving some fatty acid moieties unreacted during transesterification. This limitation could result in a lower yield of biodiesel of RMIM compared to CALB.

The findings of the current study are comparable with the results of Corrêa *et al.* [30] and Oliveira *et al.* [31] who reported the conversion of soybean oil deodorizer distillate (SODD) from Novozym 435 was 83.5% and 81.4% while 59.1% of conversion was from Lipozyme RM IM in the same reaction conditions [30]. However, current study is more competitively appealing from the economical point of view, due to use of low-cost feedstock (WCO) and low amount of enzyme loading (1.8%). The comparisons of biodiesel yield using CALB as biocatalyst is also shown in Table 2.

3.2. Triacetin Formation as By-Product

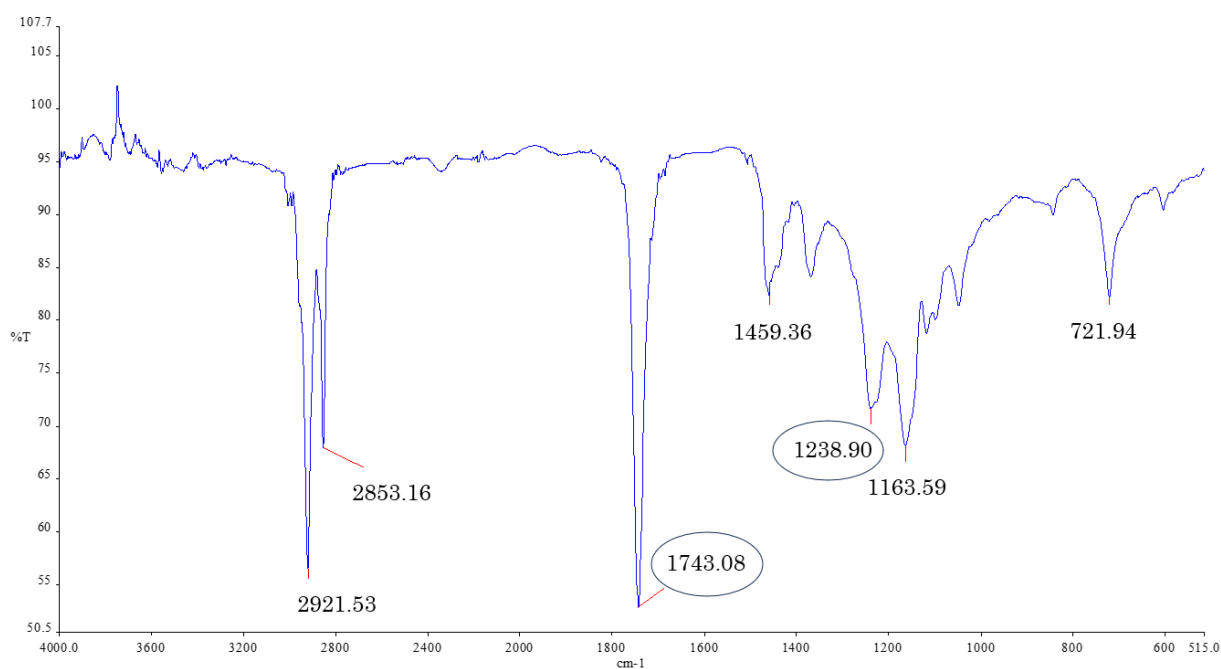Triacetin is the common by-product of the transesterification of triglycerides and methyl

Table 2. Comparison of biodiesel yield using CALB as biocatalyst.

No	Feedstock	Enzyme Loading (w/v)	Acyl Acceptor	Molar ratio	Biodiesel Yield (%)	Reference
1	Waste cooking oil	1.8%	Methyl acetate	1:12 of oil to methyl acetate	81.20	Current Study
2	Yellow horn seed oil	15%	Methanol	1:1.6 of oil to methanol	92.3	[32]
3	Castor oil	20%	Ethanol	1:10 of oil to ethanol	81.4	[31]
4	Soybean oil	30%	Methyl acetate	1:12 of oil to methyl acetate	92	[19]

acetate and has a higher added value than glycerol [33,34]. In this study, the concentration of triacetin as by-product was determined for each molar ratio of WCO to methyl acetate using CALB as biocatalyst. From Figure 4, the concentration of triacetin increases as the molar ratio of waste cooking oil to methyl acetate increases. In transesterification reactions, an excess of methyl acetate is often used to drive the reaction towards completion and improve biodiesel yield. As the molar ratio of waste cooking oil to methyl acetate increases, there is a higher concentration of methyl acetate available for reaction. This excess methyl acetate promotes the formation of triacetin as a by-product [35].

The formation of triacetin from the enzymatic transesterification of WCO was analyzed and detected by using FTIR. Figure 5 shows the IR-spectra of triacetin resulting from WCO using CALB as bio-catalyst. The dominant peaks were observed at ± 1238 cm^{-1} and ± 1743 cm^{-1}. The comparison of IR-spectra between WCO with pure biodiesel was made as shown in Figure 6, in order to determine the dominant spectrum. As may be
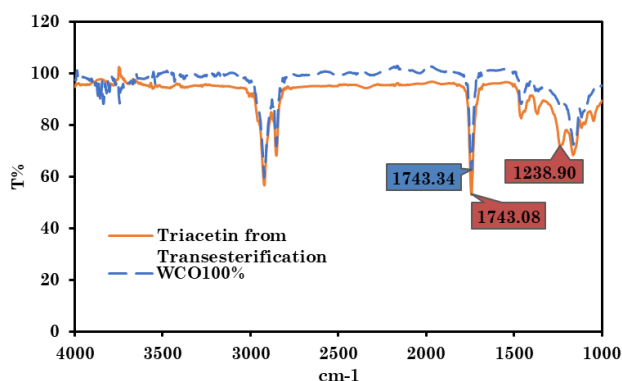
Figure 6. Comparison of IR spectra for waste cooking oil and triacetin from transesterification of waste cooking oil.
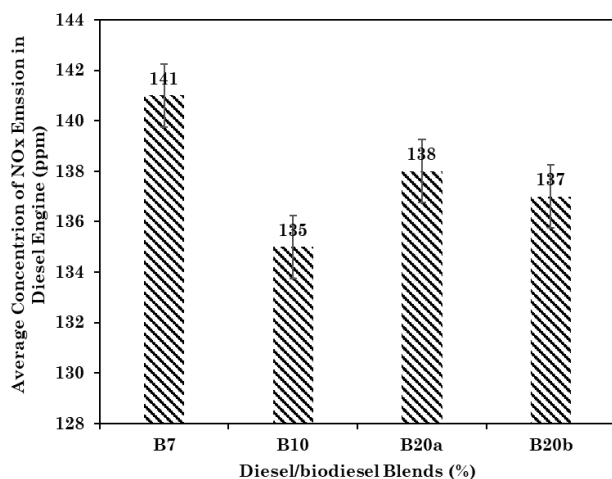
seen, a peak at ± 1743 cm^{-1} appears in both spectra of WCO and pure biodiesel. However, a peak at ± 1238 cm^{-1} is only present in the IR-spectra of transesterification of WCO with methyl acetate but absent in the IR-spectra of WCO. This proved that the triacetin is formed from the reaction and identified its characteristics peak. Similar comparisons were also reported by Pastres *et al.* [34] and Zainal *et al.* [36].

3.3. Exhaust Gas Emissions

3.3.1 Effect of biodiesel on NOx emissions

A gas analyzer (MRU Vario Plus) was used to analyze exhaust emissions such as carbon monoxide, nitrogen oxides and carbon dioxide. Several factors influence the formation of NOx in diesel engines such as the high flame temperature, peak pressure within the cylinder, nitrogen content of the fuel and the residence time of the fuel in the cylinder. Based on Figure 7, the average concentration of NOx emissions is lower when 13% of pure biodiesel is blended with industrial fuel (B7) from 141 ppm to 138 ppm. This reduction primarily occurs due to the decrease in temperature within the combustion chamber. NOx emissions tend to increase when the temperature in the combustion chamber rises [2]. However, higher NOx is emitted by blending 10% of pure biodiesel with commercial diesel fuel (B10) from 135 ppm to 137 ppm. The higher oxygen content in biodiesel's structure, coupled with elevated exhaust temperatures during combustion, contributes to the formation of nitrogen oxides (NOx) [36]. Similar cases happened which reported by Arumugam *et al.* [2], and Adaileh *et al.* [38].

3.3.2 Effect of biodiesel on CO emissions

The emissions of carbon monoxide (CO) were also measured. Based on Figure 8, the results

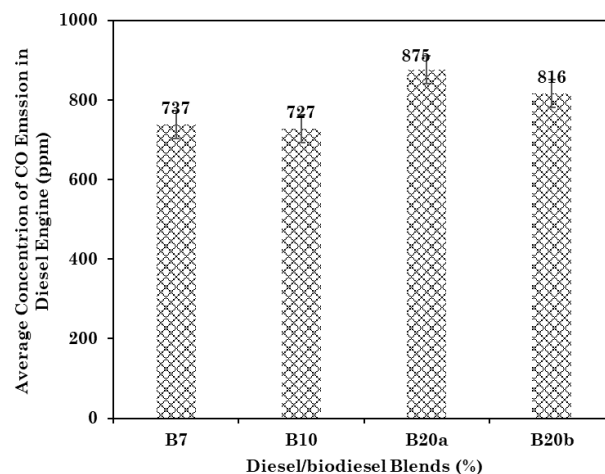Figure 7. Average concentration of NOx emissions in diesel/biodiesel blends.

Figure 8. Average concentration of CO emissions in diesel/biodiesel blends.

showed significant differences between the two scenarios (B20a and B20b). In B20a, CO emissions rose from 737 ppm to 875 ppm, indicating inefficient combustion and incomplete oxidation. In contrast, the B10 blend saw CO emissions increase from 727 ppm to 810 ppm, lower than B7 but still elevated. This considerable rise in CO concentration in the exhaust gases suggests inefficient combustion and incomplete oxidation of fuel components, which leads to more CO in the exhaust [2,37].

3.3.3 Effect of biodiesel on CO_2 emissions

The comparison between B7 and B10 blends with pure biodiesel from waste cooking oil shows different carbon dioxide (CO_2) emissions. From Figure 9, in B20a, CO_2 decreases from 1.94% to 1.67%, suggesting less efficient combustion. But B20b, CO_2 drops from 1.96% to 1.55%, indicating more efficient combustion. The value obtained for CO_2 emissions from the experiment was similar with [38] for B20 with zero load derived from waste cooking oil.

4. Conclusions

In conclusion, this study has achieved its aimed to evaluate the impact of the molar ratio of oil to methyl acetate on biodiesel production using two different immobilized lipases, *Rhizomucor miehei* lipase and *Candida antarctica* lipase B (CALB), and to assess the performance of the resultant biodiesel in a diesel engine. CALB demonstrated superior biodiesel yield compared to RMIM, particularly at a higher molar ratio of waste cooking oil to methyl acetate (1:12). A correlation between the molar ratio and the formation of triacetin was observed, confirming its presence as a by-product in biodiesel production from waste cooking oil. These findings underscore the effectiveness o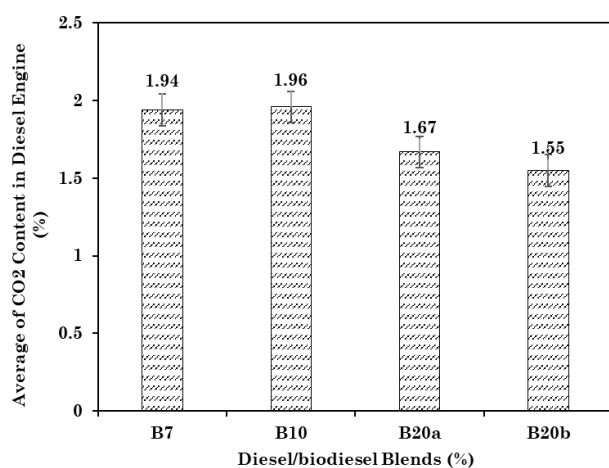f utilizing waste cooking oil as a feedstock for biodiesel production and emphasize the importance of optimizing the molar ratio for maximizing yield. Furthermore, the diesel engine performance study revealed significant advantages in lower gas emissions of carbon dioxide (CO_2) when using resulting biodiesel for biodiesel compared to commercial diesel fuel, suggesting its potential as a cost-effective and environmentally friendly alternative fuel source.

CRediT Author Statement

Nur Fatin Syafiqah: Investigation, Feedstock Collection, Resources, Data Collection, Writing Draft, Sketch Drawing, Lab Preparation; Harumi Veny: Conceptualization, Funding acquisition, Methodology, Formal Analysis, Data Curation, Writing Draft Preparation, Editing & Supervision; Fazlena Hamzah: Funding acquisition & Validation; Miradatul Najwa Muhammad Rodhi: Funding acquisition & Validation; Dhoni Hartanto: Review & Funding acquisition. Ratna Dewi Kusumaningtyas: Review & Funding acquisition; Haniif Prasetiawan: Review & Funding acquisition; Sarina Sulaiman: Review & Funding acquisition; Rozana Azrina Sazali: Review & Funding Acquisition. All authors have read and agreed to the published version of the manuscript.

Figure 9. Average of CO_2 content in diesel/biodiesel blends.

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