

UNIVERSITI TEKNOLOGI MARA

**KINETIC STUDY OF
IMMOBILIZED LIPASES IN
BIODIESEL SYNTHESIS FROM
WASTE COOKING OIL WITHIN
ULTRASONIC SYSTEM AND ITS
BIODIESEL PERFORMANCE**

**NUR FATIN SYAFIQAH BINTI
MOHAMAD NOR**

MSc

May 2026

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NUR FATIN SYAFIQAH BINTI MOHAMAD NOR

Thesis submitted in fulfilment
of the requirements for the degree of
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CONFIRMATION BY PANEL OF EXAMINERS

I certify that a Panel of Examiners has met on 20 November 2025 to conduct the final examination of Nur Fatin Syafiqah Mohamad Nor on her Masters of Science thesis entitled “Kinetic Study of Immobilized Lipases in Biodiesel Synthesis from Waste Cooking Oil Within Ultrasonic System and Its Biodiesel Performance” in accordance with Universiti Teknologi MARA Act 1976 (Akta 173). The Panel of Examiners recommends that the student be awarded the relevant degree. The Panel of Examiners was as follows:

Fauziah Marpani, PhD
Associate Professor
Faculty of Chemical Engineering
Universiti Teknologi MARA
(Chairman)

Nik Raikhan Nik Him, PhD
Associate Professor
Faculty of Chemical Engineering
Universiti Teknologi MARA
(Internal Examiner)

Fadzil Noor Gonawan, PhD
Senior Lecturer
School of Chemical Engineering
Universiti Sains Malaysia
(External Examiner)

**PROFESSOR DR HJH ZURAEDA
IBRAHIM**

Dean
Institute of Postgraduates Studies
Universiti Teknologi MARA

Date: 30 May 2026

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Name of Student : Nur Fatin Syafiqah Binti Mohamad Nor

Student ID. No. : 2022959601

Programme : Master of Science (Chemical Engineering) – CEEH750

Faculty : Chemical Engineering

Thesis Title : Kinetic Study of Immobilized Lipases in Biodiesel
Synthesis from Waste Cooking Oil Within Ultrasonic
System and Its Biodiesel Performance

Signature of Student :

Date : May 2026

ABSTRACT

Two commercial immobilized lipases, *Candida antarctica* lipase B (CALB) and *Rhizomucor miehei* lipase (RMIM) were evaluated with molar ratio of WCO to methyl acetate ranging from 1:3 to 1:12 under ultrasonic conditions at 45°C, 1.8 wt% enzyme loading and 5kHz ultrasonic frequency. As a result, CALB demonstrated the biodiesel yield of 81.2% at a 1:12 molar ratio, whereas RMIM attained a maximum yield of 16.6% at 1:3. The superior performance of CALB was attributed to its non-specific catalytic activity, enabling triglyceride conversion at all positions, in contrast to the sn-1,3 positional specificity of RMIM. The formation of triacetin as a by-product was confirmed by FTIR analysis, with a characteristic absorption peak at 1238.90 cm^{-1} . Based on its superior catalytic performance at the optimum 1:12 WCO-to-methyl acetate molar ratio, CALB was selected for kinetic evaluation. The enzymatic transesterification kinetics, involving two substrates, were described using a Ping Pong Bi-Bi mechanism with competitive inhibition by methyl acetate, and kinetic parameters were estimated via non-linear regression using Polymath 6.10 software. The fittings results were as follows: $V_{\text{max}} = 0.000026\text{M/min}$; $K_{\text{mTG}} = 0.0050\text{M}$; $K_{\text{mA}} = 0.178\text{M}$ and $K_{\text{iA}} = 0.0057\text{M}$. As a result, the model was fitted with enzyme kinetic data from the experiment and good correlations were achieved. The produced biodiesel was blended with Industrial Fuel (B7) and Commercial Diesel Fuel (B10) to form B20a and B20b blends respectively, each containing 20% biodiesel by volume. Diesel engine testing showed a reduction in engine run time with increasing biodiesel content. Exhaust emission analysis revealed a 2.13% reduction in NO_x for B20a, while a slight 1.48% increase was observed for B20b. Notably, CO₂ emissions decreased significantly, by 13.9% for B20a and 20.9% for B20b, although CO emissions increased for both blends. The observed reductions in NO_x and CO₂ demonstrate the potential of biodiesel derived from enzymatic transesterification of WCO as a more sustainable and lower-carbon alternative fuel. These findings support Malaysia's biofuel policy promoting higher biodiesel blends and reinforce the environmental viability of WCO-based biodiesel.

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LIST OF ABBREVIATIONS

Abbreviations

B7	Industrial Fuel With 7% of Biodiesel Content
B10	Commercial Fuel From Petron With 10% of Biodiesel Content
B20a	Blend of B7 With 13% of Pure Biodiesel
B20b	Blend of B10 With 10% of Pure Biodiesel
CALB	<i>Candida Antarctica</i> Lipase B
FTIR	Fourier Transform Infrared
GC/MS	Gas Chromatography-mass Spectrometry
RMIM	<i>Rhizomucor Miehei</i> Lipase
WCO	Waste Cooking Oil

LIST OF NOMENCLATURE

Nomenclatures

A	Substrate
[A]	Initial Molar Concentrations of Alcohol
Bd	Biodiesel
BSFC	Brake Specific Fuel Consumption
BTE	Brake Thermal Efficiency
BP	Brake Power
CO	Carbon Monoxide
CO ₂	Carbon Dioxide
E	Enzyme
E'	Intermediate Enzyme
E'A	Transitory Complex of Free Enzyme With Alcohol
[EA]	Concentration of Binding Enzyme With Alcohol
E'Bd	Transitory Complex of Free Enzyme With Second Product (Bd)
E'F	Transitory Complex of Intermediate Enzyme With First Product (F)
E.TG	Transitory Complex of Intermediate Enzyme With Triglyceride
EGR	Exhaust Gas Recirculation
EGT	Exhaust Gas Temperature
F	Fatty Acid
HC	Hydrocarbon

K_{mA}	Michaelis-Menten Constant for Alcohol
K_{mTG}	Michaelis-Menten Constant for Triglyceride
K_iA	Inhibitor Constant for Alcohol
NO_x	Nitric Oxide
PM	Particulate Matter
PN	Particulate Number
T_b	Brake Torque
[TG]	Molar Concentration of Triglyceride
UHC	Unreacted Hydrocarbon
V_i	Initial Reaction Rate
V_{max}	Maximum Reaction Rate in Enzymatic Reaction

CHAPTER 1

INTRODUCTION

1.1 Research Background

The current global energy system relies heavily on fossil fuels, which power major sectors such as transportation, industry, and agriculture. However, this reliance is becoming increasingly unsustainable due to the depletion of non-renewable resources, rising fuel costs, and the associated increase in greenhouse gas emissions. These emissions contribute not only to climate change but also to adverse human health effects. In response, international frameworks such as the United Nations Sustainable Development Goals (SDG 7 on affordable and clean energy and SDG 13 on climate action) and reports by the Intergovernmental Panel on Climate Change (IPCC) have driven countries to implement policies aimed at achieving substantial reductions in carbon emissions and limiting global temperature rise to 1.5 °C (Cerón Ferrusca et al., 2023). Consequently, the development of renewable and low-carbon energy sources, including biofuels, has gained significant attention.

In this context, biodiesel has emerged as a promising alternative to conventional diesel fuel. It can be produced from vegetable oils or animal fats derived from renewable biomass sources (Khan et al., 2021; Lopresto, 2025). Biodiesel can be directly used in diesel engines without major modifications, as its physicochemical properties are comparable to petroleum diesel (Dewangan et al., 2025). Environmentally, biodiesel contributes to a largely closed carbon dioxide cycle, with reported lifecycle CO₂ emission reductions of approximately 78–80% (Srikumar et al., 2024). Its oxygenated molecular structure promotes cleaner combustion, resulting in lower pollutant emissions. Additionally, biodiesel is biodegradable, enhances engine lubricity, improves performance, and supports overall sustainability (Wan Osman et al., 2024).

Despite these advantages, the sustainability and economic feasibility of biodiesel production are strongly influenced by feedstock selection. The cultivation of edible plants for biodiesel can compete with land required for food production (Zheng & Cho, 2025). Moreover, the price of edible and vegetable oils is generally higher than petroleum diesel, making large-scale production less economically viable. To address these challenges, non-edible oils such as waste cooking oil are increasingly considered

as feedstock. Being a by-product that is often discarded, waste cooking oil offers an environmentally friendly alternative while reducing production costs (Mhetras & Gokhale, 2025). Its utilization not only promotes sustainability but also mitigates issues associated with edible oil use and waste disposal (Rahman et al., 2025).

Biodiesel is typically produced through transesterification, in which oils or fats are converted into fatty acid methyl esters (FAMES) and glycerol (Zheng & Cho, 2025). Conventional chemical production generates approximately 10% glycerol by volume as a by-product, which often has limited commercial value and requires costly downstream purification. Furthermore, factors such as reactant miscibility, solvent toxicity, reaction reversibility, fuel quality, and environmental concerns motivate the exploration of alternative production processes (Rattapoltee et al., 2021). One such approach is glycerol-free biodiesel production using methyl acetate (MA) as the acyl acceptor (Wong et al., 2023). This process generates triacetin, a by-product with higher commercial value that does not deactivate enzymes and can act as a biodiesel additive (Zainal et al., 2023), improving fuel properties while eliminating costly separation steps.

Biodiesel production can be achieved through non-catalyzed, chemical-catalyzed, or enzyme-catalyzed transesterification (Norjannah et al., 2016). Non-catalyzed reactions proceed rapidly but require high temperature and pressure, resulting in high energy consumption and cost (Qadeer et al., 2025). Chemical-catalyzed reactions, either homogeneous (acid or alkali in liquid form) or heterogeneous (solid acid or base catalysts), are widely applied but face limitations such as high energy input, saponification with high free fatty acid (FFA) oils, catalyst recovery, and wastewater generation (Naseef & Tulaimat, 2025). Enzyme-catalyzed reactions using lipases offer several advantages, including high substrate specificity, mild operating conditions, low alcohol-to-oil ratio, absence of saponification, and reduced wastewater generation (Nayab et al., 2022). However, enzyme transesterification is generally limited by high cost, slow reaction rates, enzyme inhibition, and loss of activity, emphasizing the need for process optimization (Wong et al., 2023).

Factors affecting enzymatic biodiesel yield include lipase type and specificity, immobilization method, substrate composition, acyl acceptor type, and operating conditions such as temperature, molar ratio, enzyme amount, reaction time, stirring speed, pH, and water content (Ávila Vázquez et al., 2023). Enzymatic transesterification typically requires long reaction times, often between 12 and 72 hours (Norjannah et al., 2016; Vela et al., 2020) and while increasing enzyme concentration can shorten reaction

time, it is not always economically viable. Techniques such as ultrasonic irradiation have been reported to enhance reaction speed, improve biodiesel yield, and stabilize enzyme activity by promoting cavitation at the oil–alcohol phase interface, which reduces the need for mechanical agitation or high temperatures (Topare et al., 2021).

Lipases such as *Candida antarctica* lipase B (CALB), commercially available as Novozym 435, are widely used due to their high catalytic efficiency, broad substrate specificity, and stability upon immobilization (D. Li et al., 2024). Immobilization facilitates enzyme recovery and reuse, while enhancing operational stability. Other lipases, including *Rhizomucor miehei* lipase (RMIM), have also demonstrated promising catalytic activity, particularly for waste oils with high free fatty acid content. However, RMIM generally exhibits lower stability under prolonged reaction conditions and higher sensitivity to operating parameters compared to immobilized CALB, making CALB the preferred choice for many applications.

Enzymatic transesterification reactions are commonly described by a Ping–Pong Bi–Bi kinetic mechanism, in which two substrates react sequentially to form two products through enzyme–substrate intermediates (Norjannah et al., 2016). In biodiesel synthesis, this mechanism reflects the interaction between triglycerides and acyl acceptors during ester formation. The reaction rate is governed by kinetic parameters such as the maximum reaction rate (V_{max}), Michaelis–Menten constants (K_m), and inhibition constants (K_i), which collectively describe enzyme–substrate affinity and catalytic efficiency (Al-Zuhair, 2005). Determination of these parameters is essential for understanding reaction behavior and for optimizing enzymatic biodiesel production, particularly under intensified conditions such as ultrasonic systems.

Beyond production considerations, biodiesel performance in diesel engines is also influenced by exhaust emission characteristics (Wan Osman et al., 2024). Biodiesel and biodiesel–diesel blend generally reduce carbon monoxide (CO) and unburned hydrocarbons due to more complete combustion (Sethin et al., 2024). CO₂ emissions are also lower, as a portion of the carbon released is offset by carbon uptake during biomass growth. However, nitrogen oxide (NO_x) emissions show inconsistent trends, depending on feedstock, ester composition, blend ratio, and engine operating conditions (Stanescu et al., 2025). Emission profiles are further affected by engine load, speed, and duration of operation, as well as differences in testing methodologies. Therefore, standardized evaluation of CO, CO₂, and NO_x emissions under controlled conditions is essential to accurately assess biodiesel’s environmental performance.

1.2 Problem Statement

The increasing global reliance on fossil fuels during industrialization led to a surge in greenhouse gas emissions, energy insecurity, and environmental concerns. This pressing need for sustainable alternatives catalyzed research into renewable fuels, particularly biodiesel. Early studies on enzymatic biodiesel production demonstrated that lipases are capable of catalyzing transesterification reactions and follow a Ping Pong Bi-Bi kinetic mechanism as per reported by Dossat et al., (2002) and (Al-Zuhair, 2005). These foundational works established the feasibility of enzyme-catalyzed biodiesel synthesis and provided initial kinetic models. However, these studies were predominantly conducted using short chain alcohols such as methanol and were limited to conventional batch reactors with mechanical agitation. As a result, early kinetic models did not account for enzyme inhibition caused by alcohols nor address mass transfer limitations arising from the immiscibility of reactants.

Around the same period, Xu et al., (2005) investigated the kinetics of enzymatic interesterification of soybean oil with methyl acetate and developed detailed reaction models based on consecutive reversible steps and competitive substrate inhibition. This work provided foundational kinetic insights under mechanically agitated batch conditions. However, the study did not involve waste cooking oil as a feedstock or intensified reactor conditions such as ultrasonic assistance. Consequently, comprehensive kinetic studies on enzymatic interesterification of waste cooking oil using methyl acetate, particularly under ultrasonic-assisted systems, remain scarce, highlighting a clear research gap.

To overcome the slow reaction rate associated with enzymatic transesterification, ultrasonic-assisted systems were later proposed as a process intensification strategy. Ultrasonic cavitation was shown to enhance mass transfer and improve substrate–enzyme interactions. Despite these reported benefits, existing studies largely emphasize yield enhancement and process performance, with minimal attention given to reaction mechanisms and kinetic modelling under ultrasonic conditions. The influence of ultrasound on enzyme kinetics, substrate inhibition, and catalytic pathways therefore remains inadequately understood.

More recent studies employing waste cooking oil as a low-cost and sustainable feedstock have further highlighted the complexity of enzymatic biodiesel systems. However, kinetic data for enzymatic transesterification of waste cooking oil with

methyl acetate are still scarce, particularly in ultrasonic reactors. The combined effects of feedstock variability, alternative acyl acceptors, enzyme immobilization, and sonochemical conditions have not been sufficiently integrated into a unified kinetic framework.

While numerous studies have focused on improving biodiesel production efficiency, relatively fewer investigations extend their analysis to the combustion and emission behavior of the produced biodiesel fuels. Biodiesel–diesel blends have been reported to influence exhaust emissions, particularly carbon monoxide (CO), carbon dioxide (CO₂), and nitrogen oxides (NO_x); however, reported trends remain inconsistent due to variations in feedstock, fuel composition, enzyme system, and engine operating conditions. Moreover, limited studies link biodiesel production pathways and kinetic characteristics with subsequent emission performance, creating a disconnect between fuel synthesis and real-world environmental impact assessment.

Based on the reviewed literature, a clear research gap exists in the development of kinetic models for immobilized lipase-catalyzed interesterification of waste cooking oil with methyl acetate under ultrasonic-assisted conditions, as well as in relating these kinetic characteristics to exhaust emission performance. Therefore, in this study, biodiesel production from waste cooking oil using methyl acetate as an acyl acceptor is investigated using two commercially immobilized lipases, *Candida antarctica* lipase B (CALB) and *Rhizomucor miehei* lipase (RMIM), to evaluate their catalytic performance under ultrasonic assistance. The lipase exhibiting more favorable catalytic behavior is subsequently selected for detailed kinetic analysis using a PingPong Bi–Bi mechanism with competitive inhibition, and the resulting biodiesel is further evaluated in terms of CO, CO₂, and NO_x emissions under controlled engine operating conditions.

1.3 Research Objectives

The primary objective of this research is to evaluate the performance of commercial immobilized lipases as a biocatalyst for biodiesel production. Thus, biochemical characteristics were measured according to the objective below:

- a) To compare the catalytic performance of two commercially immobilized lipases in the transesterification of waste cooking oil with methyl acetate under ultrasonic conditions.

- b) To determine the kinetic parameters of commercial immobilized lipase with the best performance based on biodiesel yield in ultrasonic transesterification of waste cooking oil with methyl acetate.
- c) To investigate the efficacy of biodiesel from this study for its performance in diesel engine such as duration of running time and exhaust gas emission (CO, CO₂, NO_x) in diesel engine.

1.4 Significance of Study

The study on biodiesel production using immobilized lipase for transesterification of waste cooking oil (WCO) with methyl acetate holds significant implications for several reasons. Firstly, comparing the biodiesel yield obtained from different immobilized lipases in an ultrasonic system is essential for determining the most effective biocatalyst. Selecting the lipase that produces the highest yield ensures a more efficient transesterification process and consistent biodiesel quality

Secondly, understanding enzyme kinetic parameters, such as V , $V_{\max}$, and K_m , provides essential insights into the enzyme's efficiency and substrate affinity. Kinetic data also enable better control and prediction of the transesterification process, allowing for the fine-tuning necessary to ensure consistent product quality. Additionally, understanding these parameters can guide the development of scale efficient immobilized lipase catalysts, tailored for specific reactions or feedstocks, further enhancing the overall efficiency and versatility of the biodiesel production process.

Finally, investigating the performance of biodiesel blends in diesel engines, including operational duration and gas emissions, is essential for validating the practical applicability of biodiesel as an alternative fuel. This performance assessment helps in optimizing engine settings and modifications needed for efficient operation, ensuring engine durability and efficiency. Moreover, evaluating emissions from biodiesel blends provides critical data on the environmental benefits of biodiesel, such as reduced particulate matter, carbon monoxide, and unburned hydrocarbons compared to conventional diesel. These benefits support policy-making and regulatory frameworks promoting renewable fuels, such as Malaysia's biofuel policy, which mandates the use of biodiesel blends like B10 and higher blends such as B20. This policy is aimed at reducing the nation's carbon footprint and enhancing energy security. Reliable data on

engine performance and emissions fosters consumer confidence in biodiesel, encouraging broader adoption of biodiesel in the transportation sector and helping reduce dependence on fossil fuels, thus advancing the global transition toward sustainable energy solutions.

1.5 Limitation of Study

While the study focuses on biodiesel production from waste cooking oil using lipase enzymes, specifically *Rhizomucor miehei* lipase (RMIM) and *Candida antarctica* lipase B (CALB), in an ultrasonic system, several limitations have been identified. The research is confined for two enzymes, with RMIM representing 1,3-regiospecific lipase and CALB as a non-regiospecific lipase, which excludes the exploration of other potentially effective enzymes. Methyl acetate is used as the acyl acceptor due to its non-inhibitory effect on lipase activity and relative affordability. WCO is chosen as the primary feedstock because of its availability and cost-effectiveness even though the high in FFA contents and contains impurities and poses challenges to reproducibility and scalability. The study also utilizes ultrasonic-assisted agitation to enhance mixing and mass transfer during the reaction. Enzyme kinetics are modelled using Ping Pong Bi-Bi with substrate competitive inhibition mechanism for transesterification with methyl acetate, as proposed by Xu et al., (2004), which provides useful theoretical framework. In addition, the diesel engine performance tests are limited to evaluating running time and gas emissions of NO_x, CO and CO₂.

1.6 Scope of Study

The main scope of this study focused on biodiesel production through ultrasonic-assisted enzymatic transesterification of waste cooking oil using methyl acetate acyl acceptor. Two commercial immobilized lipases, *Rhizomucor miehei* lipase (RMIM) and *Candida antarctica* lipase (CALB), were evaluated for biodiesel synthesis at various WCO to methyl acetate molar ratios ranging from 1:3 to 1:12. RMIM was supplied with a minimum activity of 300 U/g and a lot-specific activity of 515 U/g, while CALB was supplied with a minimum activity of 1800 U/g and a lot-specific of 3367 U/g. The enzyme loading was fixed at 1.8wt% relative to the mass of WCO.

The lipase that produced the highest biodiesel yield under ultrasonic conditions was selected for further kinetic investigation. A two-substrate Ping Pong Bi-Bi mechanism with competitive inhibition was applied to describe the enzymatic transesterification reaction and kinetic parameters including $V_{\max}$, K_{mTG} , K_{mA} , and K_{iA} were determined.

The produced biodiesel was characterized using gas chromatography-mass spectrometry (GC/MS) to determine the fatty acid methyl ester (FAME) composition, while Fourier transform infrared (FTIR) spectroscopy was employed to identify functional group changes and confirm the formation of triacetin. Subsequently, the biodiesel was blended with industrial diesel (B7) and commercial diesel (B10) to prepare B7, B10, and B20 fuel blends, and the operating duration of the diesel engine was evaluated for the biodiesel blends. The scope of engine testing was limited to the evaluation of exhaust emissions, namely nitrogen oxides (NO_x), carbon monoxide (CO), and carbon dioxide (CO₂), as well as the operating duration of the diesel engine when fueled with the biodiesel blends.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Biodiesel

Energy is a fundamental driver of economic and industrial development worldwide. Among the various energy sources, fossil fuels remain the most extensively utilized due to their wide availability and established infrastructure. However, global energy consumption is projected to rise by approximately 84% by the year 2035, placing immense pressure on existing fossil fuel reserves. The continued reliance on these non-renewable sources contributes significantly to environmental degradation, including greenhouse gas emissions (Zheng & Cho, 2025). This growing concern has spurred the search for alternative and sustainable energy sources, leading to the development of biofuels such as biodiesel. Biofuels can be classified into three states which are solid (biochar), liquid (biodiesel), and gaseous (biohydrogen) (Wan Osman et al., 2024). Biodiesel itself is categorized into four generations based on the source of feedstock: the first generation is produced from edible vegetable oils; the second generation is produced from non-edible oils and WCO, the third generation is produced from algae and; and the fourth generation is produced from microalgae as illustrated in Figure 2.1.

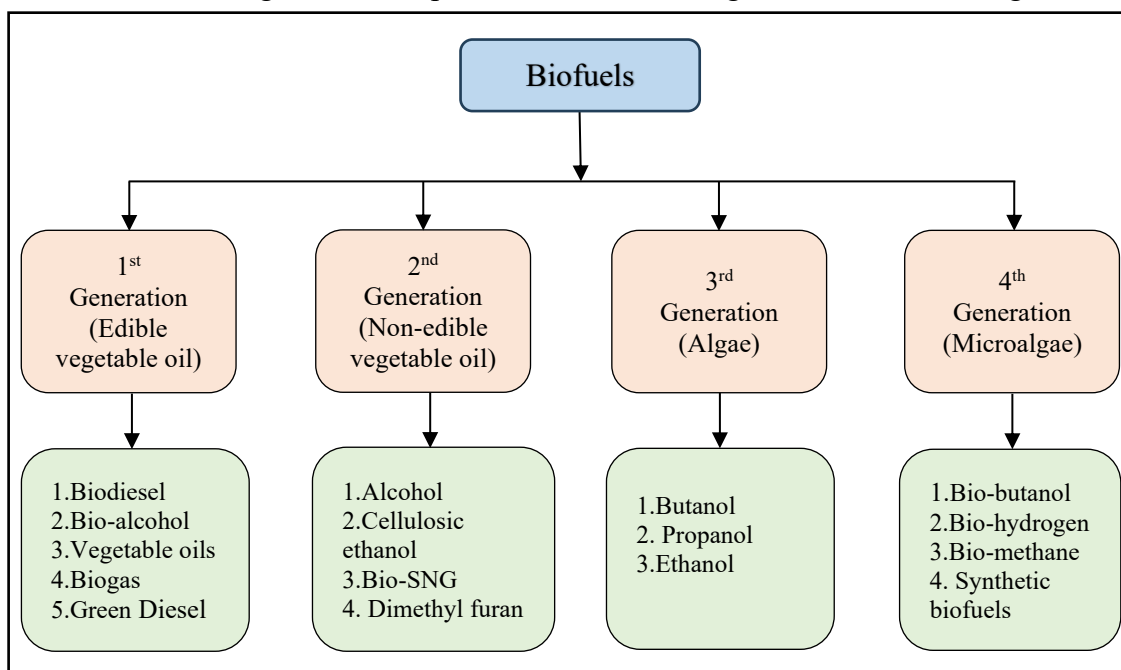


Figure 2.1 Generations of Biofuels
Source: Neupane (2023)

Growing concerns over energy sustainability and the environmental consequences of fossil fuel combustion have prompted widespread research into alternative energy sources. Among these, biodiesel has gained attention for its high biodegradability and low toxicity. It offers a promising replacement for diesel in various applications, including boilers and internal combustion engines, often requiring minimal modifications and only a slight reduction in performance. Additionally, biodiesel combustion results in significantly lower emissions of harmful substances such as sulphates, aromatic compounds, and other pollutants (Atabani et al., 2012). The increased oxygen content in biodiesel also enhances combustion efficiency, contributing to reduced emission levels.

Chemically, biodiesel consists of mono-alkyl esters of long-chain fatty acids, which are typically obtained from vegetable oils or animal fats (Da Silva et al., 2017; Thangaraj et al., 2019). It is a renewable, non-toxic, and environmentally friendly fuel source. Due to its favorable physical and chemical properties, biodiesel can be used in compression-ignition (diesel) engines with little to no modification (Lai, 2022). Compared to petroleum diesel, biodiesel offers a cleaner emissions profile by releasing lower levels of carbon monoxide, sulphur dioxide, and unburned hydrocarbons during combustion.

Biodiesel has been adopted across numerous countries, including the United States, Indonesia, Brazil, Germany, France, Italy, and Malaysia, reflecting its growing global significance as an alternative fuel. While many nations have made considerable progress in biodiesel production, there remains substantial untapped potential for expansion. As presented in Table 2.1, biodiesel output varies significantly among countries, with certain nations showing stronger development than others. Malaysia, in particular, has demonstrated a robust commitment to biodiesel development, positioning itself as a regional leader in Southeast Asia.

Table 2.1
Biodiesel Production With Different Countries

Rank	Country	Biodiesel potential (million L)	Production (\$/L)
1	Malaysia	1580	0.53
2	Indonesia	8200	0.49
3	Argentina	1177	0.62
4	USA	6100	0.70

5	Brazil	6500	0.62
6	Netherlands	2496	0.75
7	Germany	2400-2700	0.79
8	Philippines	1234	0.53
9	Belgium	1213	0.78
10	Spain	1073	1.71

Source: S&P Global 2024

Biodiesel holds significant promise as a key contributor to the global shift toward a sustainable energy mix. Historically, global biodiesel production has continued to expand dramatically from around 15,000 barrels per day in 2000 to an estimated 76.3 million tonnes in 2024 (equivalent to roughly 1.5 million barrels per day) (BioEnergyTimes, 2024), reflecting sustained growth driven by renewable fuel mandates and expanding markets worldwide. In 2024, the European Union produced approximately 18.8 million metric tons of biodiesel (BioEnergyTimes, 2024), maintaining a significant share of global output and underscoring Europe's role as a key producer in the global biodiesel industry. Mishra & Goswami, (2018), underlining the region's strong commitment to renewable fuels. This upward trajectory reflects the increasing integration of biodiesel into national energy strategies worldwide.

2.2 Sources of Biodiesel

Biodiesel is primarily produced from oils and fats derived from both plant and animal sources. Researchers have identified over 350 types of oil-bearing crops as viable sources for biodiesel feedstock (Delvi et al., 2019). In addition to these, emerging sources such as algae, microalgae, and certain fungi have also shown potential for biodiesel synthesis. Table 2.2 highlights some of the most commonly utilized raw materials in biodiesel production, adapted from Mishra & Goswami, (2018).

Table 2.2
Common Feedstocks Used for Biodiesel Production

Group	Edible Oils	Non-edible Oil	Animal Fats	Other Sources
Name of Feedstock	Soybeans	<i>Jatropha curcas</i>	Pork lard	Bacteria
	Rapeseed	Mahua	Beef tallow	Algae
	Safflower	Pongamia	Poultry fat	Microalgae
	Rice bran oil	Camelina	Fish Oil	Tarpenes
	Barley	Cotton seed	Chicken fat	Poplar
	Sesame	Karanja or honge		Switchgrass
	Groundnut sorghum	Neem		Miscanthus

Wheat	Jojoba	Fungi
Corn	Passion seed	Latexes
Coconut	Moringa	
Canola	Tobacco seed	
Peanut	Rubber seed tree	
Palm and palm kernel	Salmon oil	
Sunflower	Coffee ground	

The first generation of biofuels is conventional biofuels, which are edible feedstocks such as potato, corn, barley, soybeans, wheat, and sugarcane. The availability of the crops is the main advantage of using crops as biodiesel feedstocks. The crop plantations are also well-established in a few countries. According to USDA-Biofuels Annual Reports 2015, the usage of edible oils in biodiesel production is 30% of soybean oil, 25% of rapeseed oil, 18% of palm oil, 11% of oils from other vegetable seeds, 10% of waste cooking oil (WCO), and 6% of fats. However, consumption of edible oils is increasing, which leads to criticism over food versus fuel in utilizing edible oils as biodiesel feedstocks (Brahma et al., 2022; Naylor & Higgins, 2017). Major environmental problems are expected, such as the destruction of vital soil resources and deforestation, especially in tropical regions, including Indonesia and Malaysia, as they supply more than 75% of palm oil to the world. In addition, the price of crops has dramatically increased over the past 15 years. Hence, it is no longer suitable to use as feedstock in biodiesel production and needs to be replaced with non-edible feedstocks, which is second-generation feedstocks. The application of non-edible oils is more beneficial in biodiesel production compared to edible oils to prevent food crisis and is cost-effective (Zulqarnain et al., 2021).

2.2.1 Feedstock

The selection of an appropriate feedstock plays a pivotal role in biodiesel production, as it directly influences the yield, fuel properties, and overall production costs. Common feedstocks used in biodiesel production including plant-derived oils, animal-based fats, and recycled oils such as waste cooking oil (WCO), each presenting unique characteristics. Plant oils like soybean and canola are favored for their high triglyceride concentrations and minimal free fatty acid (FFA) content, facilitating a smoother transesterification reaction. In contrast, animal fats such as lard and tallow are more affordable but contain higher saturated fat levels, which may negatively affect the cold flow performance of the resulting biodiesel (Refaat, 2010). Microalgae have

attracted attention due to their fast growth and high lipid accumulation, offering a non-food-based alternative. However, a limitation is that many microalgae strains are obligate phototrophs, requiring light to grow, which adds complexity to cultivation (Chozhavendhan et al., 2020).

Reused oils, such as waste cooking oil (WCO) and trap grease, provide a sustainable and economical feedstock choice, as they reduce raw material costs and help minimize environmental pollution. Yet, the elevated FFA levels commonly found in these oils can trigger soap formation during base-catalyzed transesterification, thereby decreasing biodiesel yield and complicating purification. To mitigate this, pretreatment steps, typically acid esterification, are implemented to reduce FFA content before transesterification occurs (Chozhavendhan et al., 2020). The choice of feedstock is thus critical, balancing availability, cost, and processing requirements to optimize biodiesel production's economic and environmental benefits.

2.2.2 Waste Cooking Oil as Potential Biodiesel Feedstock

Waste cooking oil (WCO) is classified as a non-edible oil and is commonly derived from oils previously used in food preparation, especially frying, in restaurants and food service establishments. Chemically, cooking oil consists of glycerol esters formed from essential fatty acids and tends to be soluble only in organic solvents (Alias et al., 2018; Suzihaque et al., 2022). Improper disposal of WCO poses significant health and environmental concerns. When oxidized, the oil can generate harmful compounds potentially linked to health risks such as cancer (Ganesan et al., 2019). In many cases, WCO is discarded into drainage systems or onto the land, leading to sewer blockages and soil degradation (Alias et al., 2018). Moreover, releasing WCO into water bodies can disrupt aquatic ecosystems. According to Zare et al. (2016) utilizing waste olive oil as a biodiesel feedstock led to a reduction in carbon monoxide emissions by up to 58.9% and carbon dioxide by approximately 8.6%, showcasing its environmental benefits. Table 2.3 highlights the biodiesel production with different countries. In Malaysia alone, around 50,000 tonnes of WCO are produced annually (Kamilah et al. 2015). With its abundant availability from various food-related sectors, WCO presents itself as a promising and sustainable substitute for palm oil in biodiesel production. As its generation continues to grow, WCO holds considerable potential to serve as an effective feedstock in the renewable energy sector.

Table 2.3
Biodiesel Production With Different Countries

Country	Population of Each Country	Quantity (million tonnes/year)	References
China	1.4B	5	(De Feo et al., 2020)
Malaysia	30.75M	0.05	(Kamilah et al., 2015)
Taiwan	23.57M	0.12	(Tsai, 2019)
Indonesia	267M	0.9	(Kharina et al., 2018)
Japan	126.3M	0.45	(Newsletter, 2003)

Recent advancements in biodiesel research have evaluated numerous feedstocks, but waste cooking oil (WCO) continues to stand out due to its cost-effectiveness, widespread availability, and favorable physicochemical properties that closely resemble those of conventional diesel such as density, kinematic viscosity, acid value, and iodine value (Zare et al., 2016). However, WCO has high values of kinematic viscosity, saponification, flash point, moisture content, and free fatty acids (FFAs), which can affect biodiesel quality (Alias et al., 2018). Despite these technical barriers, the economic and environmental advantages of WCO make it a compelling option. The use of WCO can significantly reduce production costs compared to virgin oils, which are typically more expensive. Moreover, repurposing WCO diverts waste from landfills and sewage systems, helping to mitigate pollution. Importantly, this approach avoids competition with the food supply, thereby sidestepping the ethical concerns associated with edible oil-based biodiesel. In summary, while processing challenges exist, the overall benefits position WCO as a sustainable and pragmatic feedstock choice for future biodiesel development (Said et al., 2015).

2.2.3 Development of Biodiesel in Malaysia

Malaysia's venture into biodiesel development can be traced back to the early 1980s, spurred by its strategic advantage as one of the world's largest producers and exporters of palm oil. Recognizing the potential of palm oil not only as a food commodity but also as a renewable energy source, the Malaysian government took proactive steps to explore its feasibility as a sustainable biofuel feedstock. In 1982, laboratory-scale research on palm oil-based biodiesel was initiated, supported by the local palm oil industry. This early research was spearheaded by the Palm Oil Research

Institute of Malaysia (PORIM), which later merged with the Palm Oil Registration and Licensing Authority (PORLA) to form the Malaysian Palm Oil Board (MPOB), a central agency in biodiesel research and policy development (Lim & Teong, 2010). As a result of this initiative, a collaborative project between PORIM and the national oil company, Petronas, led to the establishment of a pilot plant in 1984 which capable of producing 3,000 tonnes per year of palm oil methyl ester. This milestone not only demonstrated the technical feasibility of palm biodiesel but also laid the foundation for further innovation. By 1992, Malaysia had already developed winter-grade biodiesel, an important advancement that allowed palm-based biodiesel to be used in colder climates, expanding its potential for international markets.

However, despite the early progress and technological breakthroughs of the 1980s and 1990s, the commercialization of biodiesel in Malaysia experienced a slowdown. It was not until the early 2000s, with the introduction of national energy diversification strategies, that biodiesel regained momentum. Under the Eighth Malaysia Plan (2001–2005), the government launched the Fifth Fuel Policy, which officially identified biodiesel as the "fifth fuel" in Malaysia's energy mix, alongside oil, gas, coal, and hydro (Suhara et al., 2024). This policy aimed to reduce the country's reliance on imported fossil fuels and to promote the use of indigenous renewable energy sources. The push continued under the Ninth Malaysia Plan (2006–2010), which strongly emphasized the strategic use of palm biodiesel as a cleaner alternative fuel. The pivotal moment came in 2006, with the launch of the National Biofuel Policy, which allocated around 6 million tonnes of crude palm oil (CPO) for biodiesel production. That same year marked the commissioning of Malaysia's first commercial biodiesel plant in Pasir Gudang, Johor, signifying a major leap from research and pilot testing to industrial-scale implementation. Within a short span, biodiesel production surged from 55,000 tonnes in 2006 to 230,000 tonnes by 2009, reflecting a rapid scale-up in response to both domestic policy support and international demand for renewable fuels.

Despite the early successes and the introduction of the National Biofuel Policy, the implementation of biodiesel mandates in Malaysia encountered several setbacks, particularly due to economic fluctuations. The B5 biodiesel blend mandate which required a 5% blend of palm methyl ester with petroleum diesel faced delays as crude palm oil (CPO) prices surged, increasing the cost of biodiesel production and putting pressure on government subsidies (Chung & Jusoh, 2024). These economic pressures

led to temporary considerations of lowering the mandate to B3, a move that was met with strong resistance from local biodiesel producers who were concerned about market viability and long-term industry growth. In response to this opposition, the government decided to proceed with the B5 policy, albeit with regional limitations, initially enforcing it only in specific states. This regional approach continued until 2011, allowing for gradual market adaptation while addressing subsidy concerns. A more substantial step forward came in November 2014, when the biodiesel mandate was officially raised to B7, signifying stronger governmental commitment to the biodiesel sector. This progression marked a significant milestone in Malaysia's biodiesel timeline, demonstrating a shift from experimental and pilot efforts in the 1980s to nationwide policy implementation and industry integration by the 2010s.

Malaysia has long maintained its position as one of the world's top palm oil producers, a distinction that provides the country with a unique advantage in biodiesel feedstock availability. For several decades, Malaysia was the largest global producer of palm oil, before being surpassed by Indonesia in recent years (Syafiuddin et al., 2020). Despite the abundant supply of palm oil, the journey to fully commercialize palm-based biodiesel has not been without challenges. Technical issues, such as concerns over fuel stability and engine compatibility, as well as non-technical barriers, including market competition, trade policies, and fluctuating feedstock prices, have hindered widespread adoption and export. Nonetheless, the Malaysian government remained proactive in addressing these obstacles. In 2018, further bold steps were taken with the introduction of new mandates requiring B10 biodiesel blends for the transportation sector and B20 blends for the industrial sector, including government-owned fleets. These mandates not only signalled Malaysia's commitment to environmental sustainability but also aimed to boost domestic palm oil consumption, support the rural palm oil economy, and strengthen energy security through diversification.

To further stimulate palm oil utilization and reduce reliance on fossil fuels, the Malaysian government implemented more ambitious biodiesel blend mandates beginning in 2018. The B10 biodiesel blend, comprising 10% palm methyl ester was officially introduced for the transportation sector in December 2018, while the B7 blend remained designated for industrial applications, beginning its enforcement in February 2019 (Ooi Tee Ching, 2018). In alignment with the country's broader renewable energy goals, Malaysia set a national target to raise the mandate to B20 for transportation and B10 for industrial sectors by 2020 (New Straits Time, 2019). These initiatives reflect

the government's strategy to increase domestic CPO consumption while positioning biodiesel as a sustainable fuel alternative. In support of these mandates, the government allocated RM117 million under a national agricultural budget sector (New Straits Time, 2018). A significant portion of this funding was dedicated to research and development (R&D) initiatives focused on enhancing palm oil seed productivity and improving agricultural practices, ensuring long-term feedstock sustainability and supporting smallholder farmers.

More recently, Malaysia's biodiesel programme has continued to evolve in response to sustainability goals and practical implementation challenges. The B10 biodiesel blend (10% palm methyl ester with 90% petroleum diesel) remains the mandatory national standard for the transportation sector, while higher blends are being explored in targeted applications (Reuters, 2025). Although the government had previously signalled intentions to transition the transportation sector to B20 biodiesel (20% blend), the nationwide mandate has not yet been implemented due to capital investment requirements and infrastructure constraints (Amin, 2025). Despite this, B20 use has been expanded in specific locations and sectors, including in Labuan, Langkawi, and much of Sarawak, and more recently through pilot projects such as the use of B20 for ground service vehicles and machinery at Kuala Lumpur International Airport (KLIA) (Waghmode, 2025). In line with broader environmental commitments, the government has also targeted the transition of major ports currently using B10 to B20 biodiesel, recognising its potential to reduce greenhouse gas emissions and support Malaysia's pledge to achieve net-zero carbon emissions by 2050 (Malaymail, 2025). Furthermore, under the National Agricommodity Policy (DAKN) 2021–2030, plans envisage a continuous upgrade of biodiesel blends from B15 to B20 and eventually B30 by the end of the plan period (2025), although detailed implementation timelines and infrastructure assessments are ongoing (Faiqah Kamaruddin et al., 2025; MPOB, 2026). These developments reflect a pragmatic policy trajectory that balances renewable energy ambitions with economic and technical considerations, while maintaining support for the domestic palm oil industry and its role in the national energy transition.

2.3 Production of Biodiesel via Transesterification Process

Biodiesel can be synthesized through several conversion methods, with pyrolysis, micro-emulsification, and transesterification being the most widely studied

and applied techniques. Each of these processes has unique operational characteristics, advantages, and limitations, which influence their suitability depending on the type of feedstock used and the scale of production. Among them, transesterification is the most commonly employed method due to its relatively mild operating conditions, high efficiency, and ability to produce high-quality biodiesel with properties comparable to petroleum diesel. On the other hand, pyrolysis and micro-emulsification are often explored for alternative or niche applications, particularly when dealing with low-grade or unconventional feedstocks. A comparative summary of these three methods is presented in Table 2.4.

Table 2.4
Summary of Available Process of Biodiesel Production

Process	Description	Advantages	Disadvantages	References
Pyrolysis	In pyrolysis, triglyceride (oil) is decomposed to fatty acid at high temperature with or without catalyst. Mostly used for biodiesel production if animal fats used as feedstock	The process is simple, effective and free from pollution and waste.	Must use high temperature and expensive. Biodiesel produces with low purity.	(Adewale et al., 2015c)
Transesterification	Alcohol is used as reactant. The biodiesel obtain with glycerol will be separated and purify for further process.	High conversion with low cost. The biodiesel produces closer to the petroleum diesel.	Low amount of free fatty acid and water content are needed. Generate large amount of wastewater.	
Micro-emulsification	The oils will be solubilized in a solvent and surfactant until suitable viscosity is obtained.	Simple process and free from pollution.	May lead to sticking, incomplete combustion	

Among the various methods available for biodiesel synthesis, transesterification is the most commonly utilized and commercially viable process. This method involves the alcoholysis of lipids, specifically triglycerides found in oils and fats, to produce fatty acid alkyl esters (FAAE), commonly known as biodiesel with glycerol as a valuable by-product. The use of a suitable catalyst, typically alkaline, acidic, or enzymatic, allows the reaction to be completed efficiently, often within a day under controlled conditions. Transesterification offers a significant advantage in biodiesel production as it can convert a wide range of feedstocks such as vegetable oils, animal fats, and waste cooking oils into usable fuel. The general reaction involves one mole of triglyceride reacting with three moles of a short-chain alcohol (commonly methanol) to yield three

moles of FAAE and one mole of glycerol, as illustrated in Figure 2.2. Although several alcohols, such as ethanol, propanol, and butanol, can be used, methanol is preferred due to its high reactivity, lower cost, and favorable physicochemical properties, including not forming azeotropes (Naseef & Tulaimat, 2025).

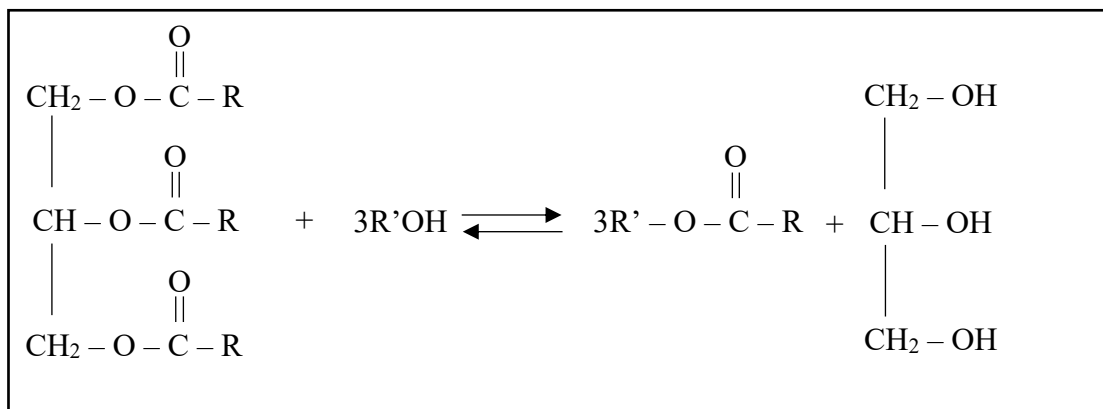


Figure 2.2 The General Chemical Reaction Depicting Transesterification of Triglyceride and Alcohol
Source: Mishra & Goswami (2018)

The transesterification process occurs in three consecutive, reversible steps. First, a triglyceride molecule reacts with methanol to form a diglyceride and one FAME molecule. This diglyceride further reacts with a second methanol molecule to produce a monoglyceride and another FAME. Finally, the monoglyceride reacts with a third methanol molecule to form the final FAME and glycerol (Naseef & Tulaimat, 2025). Because triglycerides and alcohols are immiscible under standard conditions, the system is heterogeneous. Thus, the reaction rate is greatly influenced by mass transfer between the polar alcohol and non-polar oil phases. Strategies such as mechanical stirring, heating, ultrasonication, co-solvent addition, or supercritical conditions are often employed to enhance phase interaction and improve reaction kinetics. A schematic overview of the major transesterification pathways is provided in Figure 2.3. showing the classification of catalysts into alkaline, acid, and enzymatic types. While alkaline and acid-catalyzed transesterification have been widely adopted in industrial processes for their high conversion rates, enzyme-based catalysis especially using lipases, is gaining attention for its mild operating conditions, reduced soap formation, and ease of product purification (Veluru et al., 2022). The choice of catalyst ultimately depends on the feedstock quality, particularly the free fatty acid content, and the desired process efficiency and sustainability.

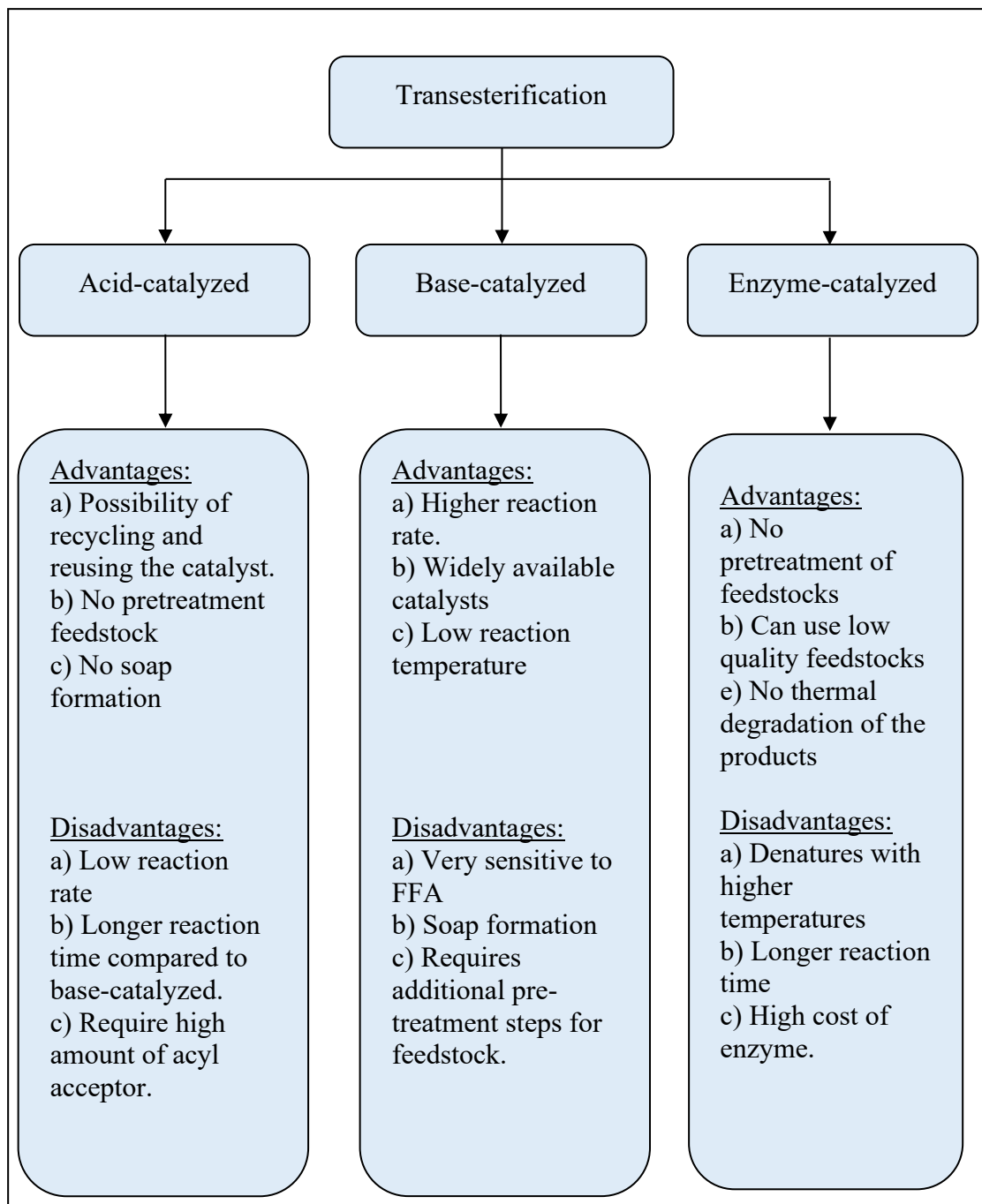


Figure 2.3 Schematic Diagram for Major Transesterification Process

2.3.1 Acid-catalyzed Transesterification

Acid-catalyzed transesterification involves the conversion of triglycerides into biodiesel (fatty acid alkyl esters) through their reaction with an alcohol in the presence of an acid catalyst. This method was historically the first approach used to produce biodiesel, initially demonstrated with palm oil, ethanol, and sulfuric acid. Acid catalysis is most commonly performed using homogeneous acid catalysts, such as sulfuric acid

(H₂SO₄), sulfonic acid, and hydrochloric acid (HCl), which are widely reported in the literature due to their strong catalytic activity (Naseef & Tulaimat, 2025).

Unlike base-catalyzed methods, acid-catalyzed transesterification is particularly suitable for feedstocks with high free fatty acid (FFA) content, such as waste cooking oil or non-edible oils. In base-catalyzed systems, high FFA levels typically result in soap formation, which hinders separation and reduces yield. Acid catalysts, on the other hand, not only catalyze the transesterification of triglycerides but also promote the esterification of free fatty acids, thereby improving the overall conversion efficiency and biodiesel yield from low-grade or impure feedstocks (Elgharbawy et al., 2021).

According to Maheshwari et al. (2022), this method typically operates at high temperatures (~100°C) and pressures (~5 bar), requiring a high molar ratio of alcohol to oil to drive the reaction to completion (Wong et al., 2023). However, the process has some limitations, including longer reaction times and the need for corrosion-resistant equipment due to the aggressive nature of acid catalysts (Elgharbawy et al., 2025). To address these challenges, heterogeneous acid catalysts have been investigated as an alternative to facilitate catalyst recovery and reuse, although their industrial application remains limited (Nayab et al., 2022). Despite these drawbacks, acid-catalyzed transesterification remains a valuable technique, especially in cases where feedstocks are unsuitable for alkaline catalysis. A summary of reported studies on acid-catalyzed transesterification in biodiesel production is tabulated in Table 2.5.

Table 2.5
Summary of Reported Studies on Acid-catalyzed Transesterification in Biodiesel Production

Feedstock	Acyl Acceptor	Catalyst	Reaction Condition	Biodiesel Yield (%)	References
Tropical Goat Fat Oil	Methanol	Homogeneous Catalyst 1. Esterification: H ₂ SO ₄ (1 wt%) 2. Transesterification: NaOH (2.5 wt%)	Esterification: 1:9 oil to methanol, Reaction Time: 1hr Temp: 65 °C 400 rpm Transesterification: 1:8 oil to methanol, Reaction Time: 1hr Temp: 65 °C ultrasound-assisted	78.5	(Cahyo Kumoro & Saeed, 2022)

Waste Cooking Oil	Methanol	Homogeneous Catalyst 4-Dodecyl benzene sulfonic acid (DBSA, organic acid)	1:9 oil to methanol, Reaction Time: 0.5hr Temp: 76 °C	90	(Athar & Zaidi, 2020)
Neem Oil	Methanol	Homogeneous Catalyst 1. Esterification: H ₂ SO ₄ (sulfuric acid) 2. Transesterification: NaOH (alkali)	Transesterification: 1:6 oil to methanol, Catalyst Loading: 1.5 wt% Reaction Time: 0.75hr ultrasound-assisted	One-step esterified oil: 83% (horn), 80% (flow cell) Two-step (esterification + transesterification): 92% (horn), 88% (flow cell)	(Joshi et al., 2018)
Corn Oil	Methanol + DME	Homogeneous Catalyst p-toluene-sulfonic acid	Catalyst Loading: 4 wt% Methanol = DME system Reaction Time: 2 hr Temp: 80°C	97.1	(Guan et al., 2009)
Soybean Oil	Methanol	Homogeneous Catalyst C ₂ HF ₃ O ₂	Transesterification: 1:6 oil to methanol, Reaction Time: 5hr Temp: 120 °C	98.4	(Miao et al., 2009)
Sunflower Oil + Oleic Acid	Methanol	Homogeneous Catalyst H ₂ SO ₄	Reaction Time: 4hr Temp: 55 °C	96	(Marchetti et al., 2007)
Deep Fried Oil (DFO) / Wild Olive Oil (WOO)	Methanol	Heterogeneous Catalyst ZrMo@KIT-6	Catalyst loading: 5 wt% (DFO), 4 wt% (WOO); 1:20 oil to methanol (DFO), 1:15 (WOO); Temp: 65 °C; Three-necked round-bottom flask	97.6 (DFO) 98.7 (WOO)	(Noman et al., 2025)
Waste Culinary Oil	Methanol	Heterogeneous Catalyst S ₂ O ₈ ²⁻ / TiO ₂ -SiO ₂ (silica-supported	1:12 oil to methanol Catalyst Loading: 5 wt%	90.7	(Abderamane Hassan et al., 2024)

		super-acid)	Reaction Time: 4hr Temp: 180°C		
Residual Fats	Methanol	Homogeneous Catalyst	1:12 oil to methanol	99.2	(Dominguez -Barroso et al., 2024)
		Methane sulfonic acid (MSA)	Catalyst Loading: 3 wt%		
		Heterogeneous Catalyst	Reaction Time: >1hr	90.5	
		SO ₃ H-C (acid carbon-based structured catalyst)	Temp: 120°C		

2.3.2 Base-catalyzed Transesterification

Base-catalyzed transesterification is the most widely adopted method in industrial biodiesel production, primarily due to its cost-effectiveness, operational simplicity, and relatively fast reaction rate. In this process, triglycerides react with a short-chain alcohol (commonly methanol or ethanol) in the presence of a basic catalyst to produce fatty acid alkyl esters (biodiesel) and glycerol as a by-product (Elgharbawy et al., 2025).

The most commonly employed base catalysts are alkaline hydroxides, such as sodium hydroxide (NaOH) and potassium hydroxide (KOH) (Nayab et al., 2022). These catalysts are homogeneous, meaning they exist in the same phase as the reactants (Králik et al., 2025), which facilitates rapid reaction rates and high conversion under moderate operating conditions. Homogeneous base catalysts are widely studied due to their operational simplicity and ability to achieve high biodiesel yields; however, they pose challenges in catalyst recovery, waste treatment, and are sensitive to feedstocks with high free fatty acids (FFA), as saponification can reduce yield and complicate product separation (Elgharbawy et al., 2025).

To overcome these limitations, heterogeneous base catalysts have been developed, typically existing as solid phases distinct from the reactants. Examples include alkaline metal oxides (e.g., CaO, BaO), metallic salts (e.g., FeSO₄, CuCl₂), and supported zeolites (Elgharbawy et al., 2025). Heterogeneous catalysts allow easier separation and reuse, reduce waste, and can minimize soap formation (Wong et al., 2023), improving the overall sustainability of the process (Gebremariam & Marchetti,

2017). Nevertheless, their generally lower activity, selectivity, and sometimes complex preparation can limit industrial adoption. Despite this limitation, base-catalyzed transesterification remains the preferred route for biodiesel synthesis due to its technical simplicity and favorable economics, particularly when using clean, refined oil sources. A summary of reported studies on base-catalyzed transesterification in biodiesel production is tabulated in Table 2.6 as below:

Table 2.6

A Summary of Reported Studies on Base-catalyzed Transesterification in Biodiesel Production

Feed-stock	Acyl Acceptor	Catalyst	Reaction Condition	Biodiesel Yield (%)	References
Castor Seed Oil	Methanol	KOH	1:12 oil to methanol Reaction Time: 24hr Temp:390°C 24 hr	84.43	(Kwon et al., 2024)
Sunflower Oil	Methanol	NaOH	1:6 oil to methanol Reaction Time: 1hr Temp:60°C	~97	(Brito et al., 2020)
Waste Cooking Oil	Ethyl acetate	NaOH	1:30 oil to methanol molar ratio Reaction Time: 3hr Temp:80°C		(Ansori & Mahfud, 2021)
Waste Cooking Oil	Methanol	KOH	1:9.4 oil to methanol Reaction Time: 2hr Temp:62.4°C	98.26	(Mohadesi et al., 2019)
Rapeseed Oil	Methyl acetate	Sodium and potassium tertbutoxide solutions in THF	1:18 oil to methanol Reaction Time: 1hr Temp:55°C	70	(Kampars et al., 2019)
Castor Oil	Methanol	KOH	1:5.4 oil to methanol Reaction Time: 2.5hr Temp:64°C	97.82	(Aboelazayem et al., 2018)
Citrullus Vulgaris Seed Oil	Methanol	NaOH	5:1 oil to methanol molar ratio Catalyst concentration: 0.13, 0.15, 0.18 g Reaction time: 90, 120,	70	(Efavi et al., 2018)

			150 min;		
			Temp: 60 °C		
Castor Oil	Methanol	KOH	1:9 oil to methanol	93	(Keera et al.,
			Catalyst Concentration:		2011)
			1wt%		
			Reaction Time: 0.5hr		
			Temp:60°C		
Cotton-seed Oil	Methanol	KOH	1:9 oil to methanol	67	(Sun et al.,
			Temp:70°C		2010)

2.3.3 Enzyme-catalyzed Transesterification

Enzyme-catalyzed transesterification has emerged as a more sustainable and environmentally benign alternative for biodiesel production, employing lipases as biocatalysts in place of conventional chemical catalysts. In contrast to acid- and base-catalyzed processes, chemical transesterification typically requires elevated temperatures and intensive downstream processing, leading to higher energy consumption and increased environmental burden. In addition, the use of chemical catalysts poses challenges in the recovery and purification of glycerol, as well as the generation of wastewater and salt-containing by-products (Lv et al., 2021). Chemical routes also impose strict limitations on feedstock quality, particularly with respect to free fatty acid and moisture content, which restrict their applicability to refined oils.

By comparison, lipase-catalyzed systems have attracted growing attention due to several inherent advantages. Lipase enzymes are capable of operating under mild temperature and pressure conditions (Nayab et al., 2022), contributing to reduced energy demand and minimizing the thermal degradation of reaction components (Wong et al., 2023).

One of the most significant strengths of this method is its capacity efficiently handle feedstocks with high levels of free fatty acids (FFAs) without leading to soap formation which is a common issue in base-catalyzed transesterification (Maheshwari et al., 2022). This makes it specially suitable for processing low-quality or waste oils, such as used cooking oil (Nayab et al., 2022; Santos et al., 2020). Furthermore, since enzymes are highly selective, the purification of the final biodiesel product is often simpler, as there is no need to neutralize chemical catalysts or remove salt by-products (Christopher et al., 2014). Despite these benefits, the widespread application of enzyme

catalysis in commercial-scale biodiesel production is currently limited by several challenges. The high cost of enzymes, along with their sensitivity to process conditions and potential deactivation over time, are key constraints (Naseef & Tulaimat, 2025). However, ongoing research in enzyme immobilization technologies and protein engineering is paving the way for improved enzyme stability, reusability, and cost-effectiveness, enhancing the practicality of this method (Nayab et al., 2022). Given its potential to address both technical and environmental limitations of conventional methods, enzyme-catalyzed transesterification is increasingly recognized as a promising strategy for future biodiesel production.

Several experimental studies have directly compared biodiesel production using chemical and enzymatic catalysts under controlled reaction conditions. In one study from Sebastian et al., (2016), using high free fatty acid rubber seed oil, chemical transesterification was performed with potassium hydroxide (KOH) as the catalyst at an oil-to-methanol molar ratio of 1:9, catalyst concentration of 1–1.5 wt%, a temperature of $55 \pm 3^\circ\text{C}$, and a reaction time of 40 minutes following a two-stage pre-treatment. In contrast, enzymatic transesterification was carried out using immobilized *Candida antarctica* lipase B (CALB) at an oil-to-methanol molar ratio of 1:4, enzyme concentration of 10 wt%, temperature of 37°C , and a reaction time of 24 hours. Unlike the chemical method, enzymatic catalysis required no pre-treatment, was tolerant to high FFA content, and allowed the use of solvents to enhance the reaction rate. Under these optimized conditions, biodiesel yields of more than 90 % were achieved with the chemical catalyst, while the enzyme-catalyzed process reached approximately 85 %. Although the chemical route provided slightly higher conversion efficiency, the enzymatic process demonstrated operational advantages such as milder reaction conditions, easier product separation, and reduced soap formation. A summary of reported studies on enzyme-catalyzed in biodiesel production is provided in Table 2.7 below:

Table 2.7
Summary of Reported Studies on Enzyme-catalyzed Transesterification in Biodiesel Production

Feedstock	Acyl Acceptor	Catalyst	Reaction Condition	Biodiesel Yield (%)	References
Used Cooking	Ethanol	Lipozyme	Temp: 40°C	96	(Aljawish et

Oil		TL IM	No prior oil refinement		al., 2025)
Waste Cooking Oil	Ethanol	CaCO ₃ – Immobilized <i>Bacillus subtilis</i> lipase	Temp: 45°C pH=8	71	(Alshehri et al., 2024)
Six Different Heterogeneous Oils	Methanol	Co-immobilized <i>Thermomyces lanuginosus</i> lipase (TLL) + <i>Burkholderia cepacia</i> lipase (BCL) on 3-GPTMS-modified Fe ₃ O ₄ magnetic nanoparticles	Reaction Time: 6hr, Extended to 12hr for multiple feedstock Optimized via RSM	92.9 (6hr) 90-98 (12hr)	(Wang et al., 2023)
Sewage sludge oil	Ethyl acetate	Novozyme 435	1:24 oil to methyl acetate Enzyme Loading: 18 wt% Reaction Time: 15hr Temp: 40°C	95.78	(Jazie et al., 2020)
Waste Cooking Oil	Methyl acetate	Lipozyme TL IM	Conventional: 1:12 oil to methyl acetate, Enzyme Loading: 6 wt% Reaction Time: 24hr Temp: 40°C Ultrasound-assisted: 1:12 oil to methyl acetate, Enzyme Loading: 6 wt% Reaction Time: 24hr	90.1 (conventional); 96.1 (ultrasound assisted)	(Subhedar & Gogate, 2016)

			Temp: 40°C		
Waste Cooking Oil	Dimethyl carbonate (DMC)	Novozym 435	1:6 oil to DMC Enzyme Loading: 10 wt% Reaction Time: 24hr	77.87	(Gharat & Rathod, 2013)
			Temp: 60°C		
Jatropha Oil	Methanol	Burkholderia cepacian	Batch: 1:3.3-3.5 oil to methanol Reaction Time: 12hr	90 (Batch) 95 (Continuous)	(Kawakami et al., 2011)
			Temp: 40°C Continuous: Flow rate 0.6mL/hr		
Soybean Oil	Ethanol	Thermomyces lanuginos	1:7.5 oil to ethanol Enzyme Loading: 15 wt% Reaction Time: 7hr	96	(Rodrigues et al., 2008)
			Temp: 31.5°C		
Rapeseed soapstock	Methanol	Pseudomonas cepacia	1:4 oil to methanol Enzyme Loading: 8 wt% Temp: 45°C	63.6	(Shao et al., 2008)
Palm Oil	Ethanol	Lipases immobilized on polysiloxane–poly (vinyl alcohol) (<i>Thermomyces lanuginosus</i> , <i>Pseudomonas fluorescens</i> , <i>Burkholderia</i>	Reaction Time: 24hr Temp: 58°C	~98	(Moreira et al., 2007)

		<i>cepacia</i> , <i>Penicillium</i> <i>camembertii</i> , <i>Candida</i> <i>antarctica</i> , porcine pancreatic)			
Rapeseed Oil	Methanol	Lipozyme TL IM	Reaction Time: 12hr Temp: 35°C	95	(Li et al., 2006)
Soybean Oil	Methanol	Lipozyme TL IM	Temp: 40°C	66	(Du et al., 2005)
Jatropha Oil	Ethanol	<i>Chromobacterium viscosum</i> lipase (free and immobilized on Celite-545)	Reaction Time: 8hr Temp: 40°C; Water addition: 1% (free), 0.5% (immobilized)	62 % (free), 71 % (immobilized), 73 % (free + water), 92 % (immobilized + water)	(Shah et al., 2004)
Sunflower Oil	Methanol	<i>Pseudomonas fluorescens</i>	Reaction Time: 48hr Temp: 40°C	97	(Soumanou & Bornscheuer, 2003)

2.3.4 Process Parameters for Biodiesel Production

The efficiency and success of biodiesel production through transesterification largely depend on a set of carefully controlled process parameters. These parameters such as reaction time, temperature, molar ratio of oil to alcohol, and catalyst concentration play an important role in determining the overall conversion efficiency and quality of the biodiesel produced (Mathiyazhagan & Ganapathi, 2011). To meet commercial and regulatory standards, it is essential to optimize each of these variables to ensure maximum yield and purity. The sensitivity of the transesterification reaction to these conditions means that even small deviations can significantly impact the reaction kinetics, product composition, and separation efficiency. Therefore, understanding and fine-tuning these parameters is crucial for developing an economically viable and environmentally sustainable biodiesel production process.

One of the most critical parameters in this process is the reaction temperature, which directly influences the rate at which the reaction occurs and the extent of conversion. In general, raising the temperature improves the solubility of the reactants, thereby accelerating the reaction and shortening the time required to reach completion (Chozhavendhan et al., 2020). Studies have shown that the optimal temperature range for most biodiesel reactions lies between 50°C and 60°C (Mathiyazhagan & Ganapathi, 2011) where sufficient energy is provided to overcome the activation energy barrier without compromising product integrity. However, exceeding this range can lead to undesirable side reactions, such as thermal degradation of fatty acid esters or the evaporation of alcohol, particularly when methanol is used as the acyl acceptor. Maintaining the reaction temperature below the boiling point of the alcohol is thus essential to prevent losses, reduce energy input, and sustain a stable reaction environment. Achieving the right balance in temperature control enhances both the biodiesel yield and the overall efficiency of the transesterification process.

Reaction time is another key variable that significantly influences the efficiency of the transesterification process. The ideal duration for the reaction is highly dependent on factors such as the type of oil feedstock, the alcohol-to-oil molar ratio, the nature and amount of catalyst used, and the operating temperature (Wong et al., 2023). Longer reaction times generally allow for a more complete conversion of triglycerides into biodiesel and by-products, as the reaction progresses toward equilibrium (Nayab et al., 2022). Higher temperatures can reduce the required reaction time by increasing the kinetic energy of the molecules, leading to more frequent and effective collisions. However, these temperatures should be kept below the boiling point of the alcohol to prevent evaporation, which would reduce the availability of alcohol and hinder the reaction. While prolonged reaction times can ensure a higher yield, they also increase the risk of unwanted side reactions and by-product formation, such as soap through saponification (Leung et al., 2010), particularly if free fatty acids are present in the oil.

Catalyst concentration also plays a pivotal role in biodiesel production, directly affecting both the reaction rate and the final yield. An insufficient amount of catalyst may result in incomplete transesterification due to limited availability of active sites, thereby reducing the biodiesel output (Yusuf et al., 2024). Conversely, using an excessive quantity of catalyst does not necessarily improve the yield. Instead, it can lead to the formation of a slurry-like mixture with increased viscosity, which hinders mass transfer and encourages soap formation, particularly in the presence of FFAs. This not

only complicates the separation of biodiesel from by-products but also lowers the overall efficiency of the process (Suzihaque et al., 2022). As a result, identifying the optimal catalyst concentration is essential, and this often depends on the specific characteristics of the feedstock and the type of catalyst employed (Yusuf et al., 2024). Careful optimization and balance of all reaction parameters (temperature, time, catalyst concentration, and alcohol-to-oil ratio) are required to achieve maximum biodiesel yield with minimal by-product formation. A summary of factors affecting biodiesel production through transesterification reaction, including reaction temperature, reaction time and catalyst concentration is Tabulated in Table 2.8:

Table 2.8
Summary of Factors Affecting Biodiesel Production Including Reaction Temperature, Reaction Time and Catalyst Concentration

Feedstock	Reaction Temperature (°C)	Reaction Time (hr)	Catalyst Amount (wt %)	Biodiesel Yield (%)	Reference
Ulva lactuca	60	1.5	NaOH, 1.50 wt%	87.8	(Binhweel et al., 2025)
Waste Cooking Oil	67.72	2.93	Eggshells/chicken bones-derived magnetic nanocatalyst (CaO/HAp/MnFe@K), 2.97wt%	99.10	(L. Li et al., 2024)
Crude Palm Oil	45	3	Immobilized CaLA, 1 wt%	80.6	(Zainal et al., 2023)
Pithecellobium dulce seed oil	45	3	NaOH, 0.5wt%	96.6	(Mahesha et al., 2022)
Waste Cottonseed Oil	45	6	Immobilized <i>Rhizopus oryzae</i> lipase, 5 wt%	98.7	(Yasvanthrajan et al., 2021)
Chicket Fat and Skin Oil	90	1	Ce-supported sulfated activated carbon (ACcs-S), 3 wt%	93	(Shobhana-Gnanaserkhar et al., 2020)
Used cooking oil	65	1.5	Fusion waste chicken and fish bones, 1.98wt%	89.5	(Tan et al., 2019)
Waste cooking Oil	65	4	CaO, 5wt%	96.74	(Borah et al., 2019)

Waste cooking oil	110	2	Sulphamic acid, 1wt%	95.6	(Gao et al., 2019)
Mixtures of refined palm oil and waste cooking oil	60	3	Biomass fly ash, 10wt%	95	(Vargas et al., 2019)
Waste Cooking Oil	50	0.5	KOH, 1wt%	94	(Sahar et al., 2018)
Cotton Seed Oil	40	24	Novozym 435, 10wt%	62.1	(Su et al., 2007)
Soybean Oil	40	14	Novozym 435, 30wt%	92	(Du et al., 2004)

In addition to process conditions, the type of feedstock used for biodiesel production also plays a crucial role in determining the final composition and quality of the biodiesel produced. Different vegetable oils yield different methyl ester profiles depending on their fatty acid composition. These profiles influence important biodiesel properties such as cetane number, cold flow behavior, and oxidative stability (Chozhavendhan et al., 2020). Table 2.9 summarizes the fatty acid methyl ester (FAME) composition in biodiesel derived from various vegetable oil sources, as reported in the literature.

Table 2.9
Composition of Methyl Ester in Biodiesel from Different Types of Vegetable Oils

Type of Vegetable Oils	Compound (Methyl ester)	Composition (%)	References
Micro-algae	9-octadecenoic Acid Methyl Ester	20.65	(Verma et al., 2024)
	Linoleic Acid Ethyl Ester	9.67	
	Hexadecanoic Acid, Methyl Ester	6.26	
Cotton Seed Oil	Nonanoic Acid Methyl Ester	0.20	(Usman et al., 2016)
	Octadecanoic Acid Methyl Ester	3.49	
	Pentanoic Acid Methyl Ester	11.53	
	Hexadecanoic Acid Methyl Ester	0.54	
	Octadecadienoic Acid Methyl Ester	14.23	
	Octadecadienoic Acid Methyl Ester	17.72	

	Octadecadienoic Acid Methyl Ester	7.32	
	Oleic Acid Acid Methyl Ester	3.41	
	Eicosadienoic Acid Methyl Ester	1.81	
	Octadecanoic Acid Methyl Ester	1.49	
	Methyl Ricinolate Acid Methyl	1.85	
	Eicosanoic Acid Methyl Ester	3.47	
	Hexadecanoic Acid Methyl Ester	7.97	
	Octadecenoic Acid Methyl Ester	15.62	
Waste	Octadecanoic Acid, Methyl Ester	62.02	(Haq et al., 2021)
Cooking	Octadecadienoic Acid, Methyl Ester	10.85	
Oil	Hexadecadienoic Acid, Methyl Ester	8.91	
	Eicosadienoic Acid, Methyl Ester	6.45	
	Pentadecanoic Acid, Methyl Ester	4.23	
	Tetradecanoic Acid, Methyl Ester	4.10	
	Heptacosanoic Acid, Methyl Ester	3.92	
Sunflower	9-Octadecadienoic acid, Methyl	54.29	(Varghese et al., 2017)
Oil	Ester		
Palm Oil	Methyl Tridecanoate	1.58	(Fadlly et al., 2021)
	Methyl Heptadecanoate	0.24	
	Methyl Oleate	20.85	
Canola	Hexadecanoic Acid, Methyl Ester	4.60	(Shamsudin et al., 2022)
Oil	9-octadecenoic acid (z), Methyl Ester	64.30	
	9,12,15-octadecatrienoic acid, (z,z,z),	7.60	
	Methyl Ester		
Soybean	Hexadecanoic Acid, Methyl Ester	8.95	(Shamsudin et al., 2022)
Oil	9-octadecenoic acid (z), Methyl Ester	22.95	
	9,12,15-octadecatrienoic,	5.95	
	acid, (z,z,z), Methyl Ester		
Greenseed	Hexadecanoic Acid, Methyl Ester	4.36	(Shamsudin et al., 2022)
Canola	9-octadecenoic acid (z), Methyl Ester	6.92	
Oil	9,12,15-octadecatrienoic acid, (z,z,z),	6.76	
	Methyl Ester		
Canola	Methyl Palmitate	10.0	(Park et al., 2019)
and	Methyl Stearate	1.5	
peanut oil	Methyl Oleate	64.9	

Methyl Linoleate	20.1
Methyl Linolenate	1.9

2.4 Oil to Acyl Acceptor Molar Ratio

In transesterification-based biodiesel production, the molar ratio between oil (triglycerides) and the acyl acceptor (such as methanol or methyl acetate) is one of the most critical parameters affecting the efficiency and yield of the process. This ratio determines the chemical equilibrium of the reaction and plays a decisive role in pushing the conversion of triglycerides into fatty acid esters (biodiesel) to completion. Since transesterification is a reversible reaction, an excess of the acyl acceptor is often used to shift the equilibrium toward product formation, in accordance with Le Chatelier's principle.

Typically, a higher molar ratio of acyl acceptor to oil increases biodiesel yield by enhancing the contact between reactants, thereby promoting more complete triglyceride conversion. However, excessively high ratios may introduce new challenges, such as greater difficulty in downstream separation and purification processes, particularly when dealing with volatile acyl acceptors like methanol or methyl acetate. These inefficiencies can lead to higher energy consumption and increased production costs, offsetting the benefits of improved conversion.

The optimal molar ratio varies depending on the type of catalyst (chemical or enzymatic) and reaction conditions. For base-catalyzed systems, a commonly recommended ratio is 6:1 (methanol:oil) to ensure adequate conversion without excessive methanol use (Refaat., 2010). In contrast, enzyme-catalyzed reactions, especially those using immobilized lipases, often require significantly higher molar ratios, such as 12:1, to overcome mass transfer limitations and enzyme inhibition (Vicente et al., 2004). These elevated ratios help maintain reaction efficiency and minimize the potential inhibitory effects of by-products like glycerol or acyl acceptors on enzyme activity. Ultimately, the selection of a suitable molar ratio should be carefully tailored based on the feedstock characteristics, catalyst type, and economic considerations of the production process. A well-balanced molar ratio contributes not only to high biodiesel yields but also to cost-effective and sustainable process operation. A comparison of biodiesel synthesis using different types of feedstocks, acyl acceptors and molar ratios is provided in Table 2.10.

Table 2.10
Summary of Biodiesel Synthesis Using Different Types of Acyl Acceptor

Feedstock	Acyl Acceptor	Reaction Condition	Biodiesel Yield (%)	References
Soybean Cooking Oil Waste	Methanol	1:6 oil to methanol; Reaction Time: 36hr Temp: 37°C	96	(Adebami et al., 2025)
Waste Cooking Oil	Ethanol	1:10 oil to ethanol; Reaction Time: 12hr Temp:45 °C	71	(Alshehri et al., 2024)
Waste Cooking Oil	Methanol	1:10 oil to methanol; Reaction Time: 24hr Temp:55 °C	97.66	(Ben Bacha et al., 2022)
Sludge Palm Oil	Methanol	1:3 oil to methanol; Reaction Time: 4hr Temp:40 °C	91.30	(Muanruksa & Kaewkannetra, 2020)
Oleaginous Yeast	Methanol (with n-hexane co-solvent)	Reaction Time: 4hr Temp:35 °C	98.12	(Cao et al., 2021)
Castor Oil	Ethanol	1:10 oil to ethanol; Enzyme Loading: 20wt% Reaction Time: 6hr Temp: 65°C	98	(De Oliveira et al., 2004)
Soybean Oil	Methanol	1:3.4 oil to methanol; Reaction Time: 6.3hr Temp:36.5 °C	92.2	(Shieh et al., 2011)
Refined Soybean Oil	Methanol	1:3 oil to methanol; Enzyme Loading: 4 wt% Reaction Time: 48hr	95.9	(Watanabe et al., 2002)
Crude Soybean Oil	Methyl Acetate	1:12 oil to methyl acetate; Enzyme Loading: 30 wt% Reaction Time: 14hr	92	(Watanabe et al., 2002)
Olive Oil	Dimethyl Carbonate (DMC)	1:3 oil to DMC; Reaction Time: 24hr n-heptane	81.2	(Su et al., 2007)
Rapeseed Oil	Dimethyl Carbonate (DMC)	1:3 oil to DMC; Reaction Time: 24hr	78.5	(Su et al., 2007)
Cotton Seed Oil	Dimethyl Carbonate (DMC)	1:3 oil to DMC; Reaction Time: 24hr	96.4	(Su et al., 2007)
<i>Jatropha curcas</i> Oil	Ethyl Acetate	1:12 oil to ethyl acetate; Enzyme Loading: 10 wt%	91.3	(Modi et al., 2007)

Reaction Time: 12hr				
Temp:50 °C				
Tallow	Iso-butanol	1:3 oil to iso-butanol;	98.5	(Nelson et al., 1996)

2.4.1 Methyl Acetate as Acyl Acceptor

Methyl acetate ($\text{CH}_3\text{COOCH}_3$) also known as acetic acid methyl ester is an organic ester commonly used as an industrial solvent such as adhesives, paints and nail polish removers. Beyond its traditional roles, methyl acetate has recently gained attention in the field of biodiesel production (Usai et al., 2010), particularly in enzymatic transesterification processes, as a promising alternative acyl acceptor. Figure 2.4 shows the molecular structure of methyl acetate, illustrating its ester functional group which plays a key role in acyl transfer during enzymatic reactions.

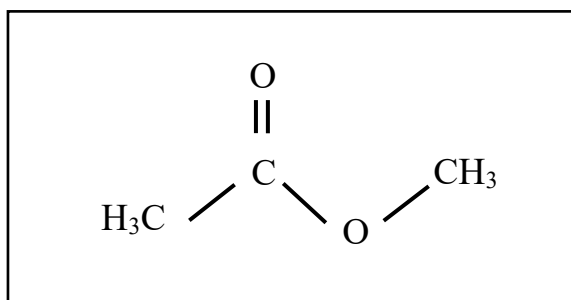


Figure 2.4 Molecular Structure of Methyl Acetate

Conventionally, biodiesel is synthesized using short chain alcohols like methanol or ethanol as acyl acceptors. While effective, these alcohols pose several drawbacks when used in enzymatic systems. One major challenge is their tendency to inactivate lipase enzymes. Short-chain alcohols can denature the protein structure of lipases by disrupting their hydrophobic interactions, ultimately reducing the catalytic efficiency and leading to enzyme deactivation (Huang & Yan, 2008). Furthermore, the transesterification of triglycerides with methanol or ethanol produces glycerol as by-product (Naseef & Tulaimat, 2025). Glycerol tends to accumulate around the active sites of immobilized lipases, forming a physical barrier that impedes substrate access and diminishes enzymatic activity over time (Wong et al., 2023).

At the molecular level, acyl acceptors participate in lipase-catalyzed transesterification by attacking the acyl-enzyme intermediate formed during the reaction. This attack releases the fatty acid as a methyl ester while regenerating the active enzyme for subsequent catalytic cycles. The type and concentration of acyl

acceptor significantly influence reaction efficiency. Short-chain alcohols, such as methanol, may inhibit the enzyme by competitively binding to the active site or destabilizing the protein structure. In contrast, non-alcohol acyl acceptors like methyl acetate are less inhibitory (Qadeer et al., 2025). In addition to forming fatty acid methyl esters (FAME), methyl acetate acetylates the glycerol backbone to produce triacetin instead of free glycerol (Usai et al., 2010). Triacetin is miscible with biodiesel and does not accumulate on the immobilized enzyme surfaces, preventing obstruction of the active sites and allowing the enzyme to maintain catalytic performance (Duraiarasan et al., 2013).

The use of methyl acetate also offers additional operational benefits. Its lower polarity compared to methanol contributes to improved solubility of hydrophobic substrates like triglycerides in the reaction mixture, reducing mass transfer limitations (Salaheldeen et al., 2021). During transesterification, polar alcohols may alter the microenvironment, whereas methyl acetate maintains favorable reaction conditions, facilitating effective substrate interaction with the enzyme. Moreover, the triacetin by-product is non-polar, low-viscosity, and does not interfere with enzymatic activity. Its formation prevents glycerol accumulation, which could otherwise impede enzyme function, and enhances the overall efficiency of the reaction (Wong et al., 2023).

Another notable advantage of methyl acetate is its ability to promote higher biodiesel yields in ultrasonic-assisted or solvent-free systems due to its reduced inhibitory effects on the enzyme (Qadariyah et al., 2026). The combination of these benefits has led to growing interest in methyl acetate as a green and sustainable acyl acceptor for biodiesel production from waste oils and fats. Figure 2.5 illustrates the transesterification mechanism consisting of three reversible reactions, showing the stepwise conversion of triglycerides into biodiesel and triacetin when methyl acetate is used as the acyl acceptor.

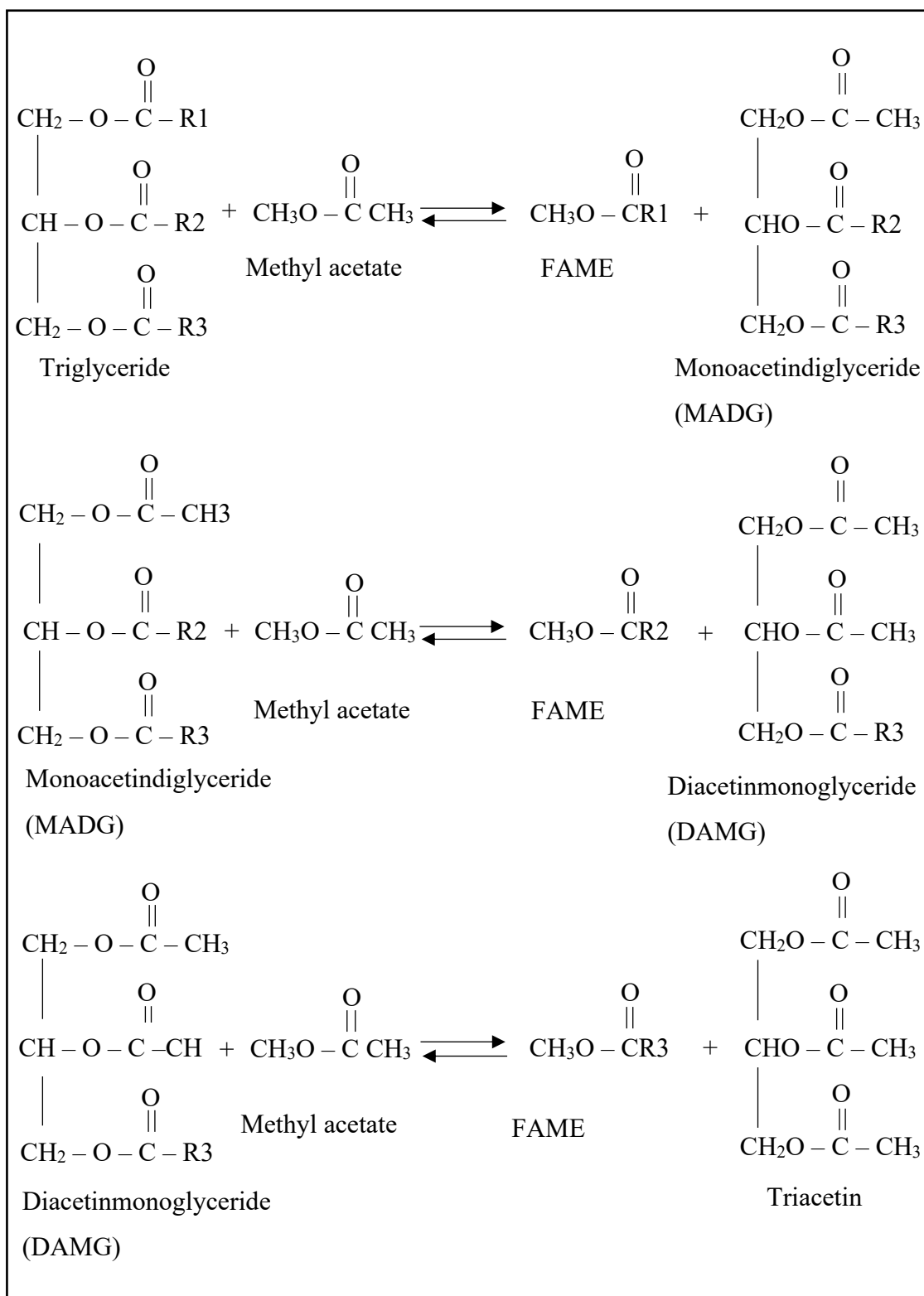


Figure 2.5 Transesterification of Triglyceride with Methyl Acetate (Interesterification)

Unlike glycerol, the by-product of conventional methanolysis, triacetin does not adsorb onto the enzyme surface or block pores of immobilized lipases. Furthermore, triacetin has additional value as a gelatinizing agent and plasticizer in the pharmaceutical and cosmetic industries, enhancing the economic feasibility of biodiesel

production (Zainal et al., 2023). Table 2.11 summarizes previous studies involving the use of methyl acetate as an acyl acceptor in biodiesel production from various feedstocks and catalysts. This comparison helps contextualize the advantages and performance of different systems, supporting the rationale for using methyl acetate in this study.

Table 2.11
Biodiesel Production Using Methyl Acetate As Acyl Acceptor From Various Feedstock and Catalyst

Feedstock	Catalyst	Reaction Conditions	Biodiesel Yield (%)	References
Coconut Oil	Eggshell-derived CaO (heterogeneous catalyst), calcined at 900°C for 2hr	Ultrasonic-assisted interesterification; 55°C; 1:9.7 oil to ethyl acetate; 0.1 hr	81.17	(Qadariyah et al., 2026)
Waste Cooking Oil	KOH	1:24 oil to methyl acetate; 50°C; 0.4 hr; RSM method	92.5	(Qadeer et al., 2025)
Babassu Oil	H ₂ SO ₄	Ultrasonic-assisted interesterification; 1:60 oil to methyl acetate; 3 hr	95.4	(Priebe et al., 2024)
Ceiba pentandra Oil	Nendran banana peduncle ash (NBPA) heterogeneous catalyst derived from agricultural residue	Two-step process (esterification – transesterification); Continuous ultrasound assisted reaction; 1:10 oil to methyl acetate; 0.75hr	94.6	(Balajii & Niju, 2023)
Rapeseed Oil	SnO@ γ -Al ₂ O ₃ composite catalyst	Interesterification; methyl acetate; Ultrasonic-assisted catalyst synthesis; 210°C; 0.5 hr	38% higher FAME yield compared to commercial catalyst	(Prestigiacomo et al., 2022)
Soybean Oil	Calcium oxide (CaO)	Interesterification; Methyl acetate; 1:40 oil to methyl	62.3 wt% of FAME content	(Nunes & Castilhos, 2020)

		acetate; 325°C; 10wt% catalyst		
Soybean Oil	Calcined Mg–Al hydrotalcite	1:50 oil to methyl acetate, 200°C, 4hr	95.9	(Dhawan et al., 2021)
Karanja Oil	γ -Alumina (γ - Al ₂ O ₃)	Ultrasound- assisted interesterification; 1:9 oil to methyl acetate; 0.83 hr; 50°C	69.3	(Kashyap et al., 2019)
Cottonseed oil	Potassium methoxide	Ultrasound- assisted interesterification; 1:14.87 oil to methyl acetate; 1.17 wt% of catalyst loading; RSM method	98.12	(Medeiros et al., 2018)
Rapeseed Oil	Sodium methoxide	Chemical interesterification with alkyl acetates; 1:36 methyl acetate to oil, 1hr, 55°C	93	(Sustere et al., 2016)
Oil from wet cells of marine microalga, <i>Chlorella salina</i>	Immobilized enzymes on magnetic nanoparticles	Interesterification with immobilized lipase; 1:12 methyl acetate to oil, 48hr, 45°C	93.56	(Duraiarasan et al., 2016)
Waste Cooking Oil	Lipozyme TLIM	Ultrasound- assisted transesterification, 1:12 oil to methyl acetate; 3 wt% of enzyme loading; 40°C; 3hr	96.1	(Subhedar & Gogate, 2016)
Oil from microalgae	Encapsulated lipase from <i>Bacillus</i> sp. S23, immobilized in	Enzymatic interesterification, solvent free; 1:12 oil to methyl	95.68	(Surendhiran et al., 2015)

	sodium alginate beads	acetate; 35°C , 60hr; 250 rpm		
Oil from marine microalga <i>Chlorella salina</i>	Immobilized whole-cell yeast <i>Rhodotorula</i> <i>mucilaginosa</i> MTCC8737 on sugarcane bagasse	Enzymatic transesterification; 1:12 oil to methyl acetate; 40°C; 60 hr; 250 rpm	85.29	(Surendhiran et al., 2014)
Waste Cooking Oil	Potassium methoxide	1:12 methyl acetate to oil, 0.5hr, 40°C	90	(Maddikeri et al., 2013)
Waste Canola Oil	Novozym 435	1:14 methyl acetate to oil, 24hr, 48°C	72	(Ruzich & Bassi, 2010)

2.5 Lipases in Biodiesel Production

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are among the most extensively utilized enzymes in biotechnology, particularly in sectors such as pharmaceuticals, food processing, and renewable energy (Godoy et al., 2022). Their role in biodiesel production has garnered considerable attention due to their catalytic efficiency under mild conditions and broad substrate specificity (Naseef & Tulaimat, 2025). These enzymes possess the unique ability to catalyze a range of bio-conversion reactions, including hydrolysis, alcoholysis, aminolysis, esterification, and interesterification, depending on the nature of the substrates and reaction environment.

The enzymatic production of biodiesel represents a successful application of lipase-catalyzed transesterification. This reaction typically occurs in two steps: first, the hydrolysis of the ester bonds in triglycerides to release free fatty acids (FFAs) and diglycerides; second, the esterification or transesterification of these intermediates with an acyl acceptor such as methanol or methyl acetate, resulting in the formation of fatty acid methyl esters (FAMEs) and glycerol as a by-product.

Lipases can be sourced from various biological origins, including plants, animals, and microorganisms. Among these, microbial lipases, particularly those derived from bacteria and fungi are favored for industrial-scale applications (Mussakhmetov & Silayev, 2025). Their advantages include rapid growth rates, ease of

cultivation, consistent enzyme yield, and amenability to genetic and process optimization (Ribeiro et al., 2011). Moreover, microbial enzymes tend to exhibit desirable stability and activity profiles, which are critical for robust catalytic performance.

A wide array of microbial lipases has been studied and employed in biodiesel synthesis. Notable examples include enzymes from *Candida antarctica*, *Candida rugosa*, *Thermomyces lanuginosus*, *Rhizomucor miehei*, *Mucor miehei*, *Aspergillus niger*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Chromobacterium viscosum*, *Rhizopus oryzae*, and *Streptomyces* species. Each of these enzymes exhibits varying degrees of substrate specificity, positional selectivity (regiospecificity), and stability, which influence their effectiveness in transesterification processes. Based on their substrate preferences, lipases are generally categorized into three groups:

- 1,3-specific lipases, which selectively target the ester bonds at the first and third positions of the triglyceride backbone.
- Fatty acid-specific lipases, which favor certain chain lengths or saturation levels.
- Non-specific lipases, which can act at all positions of the glycerol backbone without preference.

2.5.1 Mechanism of Lipase in Enzymatic Transesterification

Lipases are a class of enzymes that play a crucial role in the enzymatic transesterification process by catalyzing the conversion of TAGs at the oil-water interface and in the presence of an acyl acceptor (such as methanol or methyl acetate), to carry out transesterification reactions effectively. This process involves a catalytic triad composed of aspartic acid, histidine, and serine, forming a charge-relay system. Initially, histidine facilitates the deprotonation of serine alcohol, enhancing its nucleophilicity. This results in the formation of an oxyanion on serine, which attacks the carbonyl carbon of the substrate, forming a tetrahedral intermediate. Aspartic acid and histidine stabilize this oxyanion through hydrogen bonding. Subsequently, the proton is transferred from histidine to the diglyceride, leading to its release. The resulting serine ester undergoes transesterification with alcohol, completing the reaction. Histidine then deprotonates the alcohol molecule, generating an alkoxide anion. This hydroxide attacks the carbonyl carbon, forming another tetrahedral intermediate, from which a free fatty acid is released. The serine oxygen re-establishes

hydrogen bonding with histidine, while aspartic acid assists in stabilizing the positive charge on histidine during its protonation. Overall, this mechanism elucidates how lipases efficiently catalyze the breakdown of TAG molecules into glycerol and fatty acids. The schematic illustration of the lipase-catalyzed transesterification mechanism, adapted from Jegannathan et al., (2008) as presented in Figure 2.6 as below:

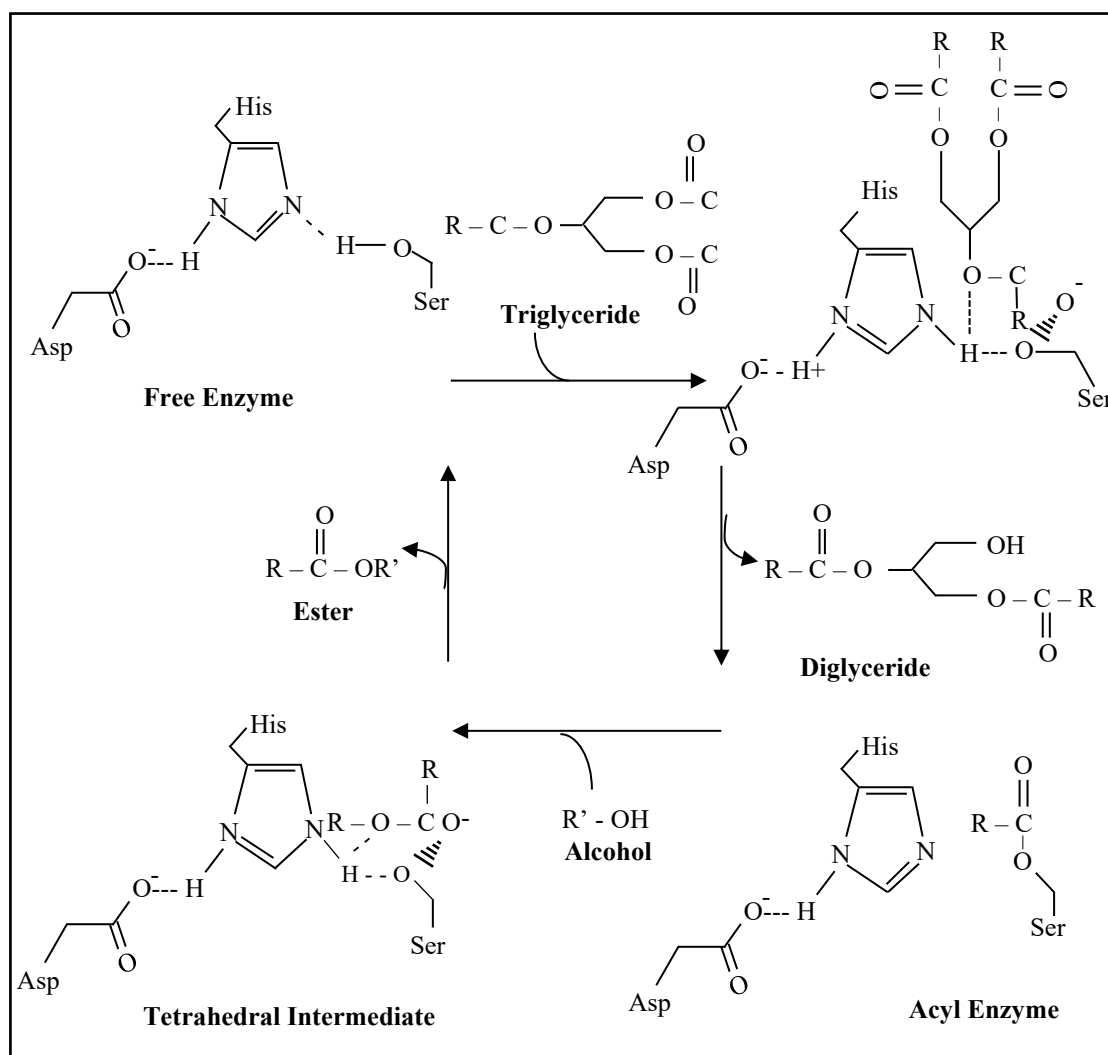


Figure 2.6 Mechanism of Lipase in Transesterification

2.5.2 Immobilization of Lipases

Although free lipases can catalyze transesterification reactions effectively, their instability, difficulty in recovery, and high cost hinder their direct application in industrial biodiesel production. Immobilization of lipases addresses these challenges by enhancing their operational stability, activity, and reusability. Immobilized lipases are enzymes physically or chemically attached to solid carriers, allowing for repeated use and easier separation from the reaction mixture. This technique not only improves

enzyme resistance to extreme pH, temperature, and solvents but also maintains catalytic activity over multiple cycles. In industrial applications, including food, pharmaceutical, and cosmetic industries, immobilized enzymes offer economic and environmental benefits due to reduced biocatalyst consumption and waste generation (Facin et al., 2019). In the context of biodiesel production, immobilization offers an added advantage by minimizing product inhibition, particularly when using hydrophobic supports that improve substrate accessibility. Furthermore, immobilized lipases show enhanced selectivity toward specific substrates, which is crucial when dealing with waste cooking oil containing mixed fatty acid compositions.

2.5.3 *Rhizomucor miehei* Lipase (RMIM)

Among many lipases utilized in enzymatic transesterification, *Rhizomucor miehei* lipase (RMIM) stands out due to its high catalytic activity, substrate specificity, and operational stability. Formerly known as *Mucor miehei*, *Rhizomucor miehei* is a well-known filamentous fungus widely recognized for producing a commercially available lipase with extensive industrial applications. RMIM is classified as an sn-1,3-specific lipase that is capable of hydrolyzing ester linkages at the sn-1 and sn-3 positions of glycerol (Tian et al., 2021). This specificity is particularly beneficial in biodiesel production as it enhances the conversion efficiency by selectively acting on accessible ester bonds without complete breakdown of the entire glyceride molecule, thereby improving product yield and process control. Structurally, the molecular size of RMIM is 31.6 kDA, composed of 269 amino acids (Tian et al., 2021). Like many serine hydrolases, its catalytic activity is driven by a conserved catalytic triad of Ser-His-Asp. These residues are strategically positioned within the active site to facilitate nucleophilic attack and proton transfers during the reaction mechanism.

A defining structural feature of RMIM and many lipases is the presence of a “Lid” structure, which plays a crucial role in the enzyme’s function. This lid covers the active site under aqueous conditions but opens at the oil–water interface, exposing the hydrophobic catalytic center. The outer surface of the lid is hydrophilic, while the inner surface and the catalytic pocket are hydrophobic, an arrangement that enables interfacial activation (Khan et al., 2007). When the enzyme encounters a hydrophobic substrate, such as triglyceride in an immiscible oil phase, the lid undergoes a conformational change, facilitating substrate access to the active site. This unique mechanism allows

RMIM to function efficiently under biphasic conditions typical of transesterification systems. The amphiphilic nature and structural adaptability of RMIM make it highly suitable for biodiesel production, especially when dealing with complex feedstocks like waste cooking oil. Its regioselectivity and substrate affinity further contribute to its performance in esterification and transesterification reactions, reducing side reactions and enhancing selectivity toward fatty acid alkyl esters. Table 2.12, highlighting the effectiveness of RMIM as a biocatalyst in enzymatic biodiesel synthesis.

Table 2.12
Biodiesel Synthesis Yield Using Immobilized *Rhizomucor miehei* Lipase as Biocatalyst

Lipase Source	Condition/ Method	Feedstock	Molar Ratio	Yield (%)	References
Lipozyme RM IM	-	Fish and chicken waste fat	1:4 oil to isoamyl alcohol	98%	(Gumbyte et al., 2025)
<i>Rhizomucor miehei</i>	Silica	Waste cooking oil	1:6 oil to methanol	91.08	(Chen et al., 2009)
Mixed of <i>Candida rugosa</i> and <i>Rhizomucor miehei</i>	Poly- hydroxybutyrate	Waste cooking oil	1:6 oil to methanol	96.5	(Binhayeeding et al., 2020)
<i>Rhizomucor miehei</i>	Macroporous anion exchange resin	Sunflower oil	1:6 oil to methanol	70	(Calero et al., 2019)
Mixed of <i>Candida antarctica</i> lipase B and <i>Rhizomucor miehei</i>	Macroporous resin (under oil optimal condition in isooctane system)	Soybean oil	1:4 oil to methanol	99.2	(Li et al., 2018)
<i>Rhizomucor miehei</i>	Immobilized on yeast cell wall	Soybean oil	1:4.5 oil to methanol	83.14	(Huang et al., 2012)
<i>Rhizomucor miehei</i>	Immobilized on biomass oil support particles	Soybean oil	1:6 oil to methanol	94.3	(Jin et al., 2013)
Mixed of <i>Candida antarctica</i>	Immobilized on epoxy functionalized	Palm oil	1:9 oil to methanol & 1:2.5 of RMIM	78.3	(Shahedi et al., 2019)

lipase B and <i>Rhizomucor miehei</i>	silica gel		to CALB		
<i>Rhizomucor miehei</i>	Semi-rational design produce mutants	Colza oil	1:3 oil to methanol	N218-90.46 N260-88.75	(Tian et al., 2021)
<i>Rhizomucor miehei</i>	Immobilized on an ion exchange resin	Sunflower oil	1:3 oil to methanol	>80	(Soumanou & Bornscheuer, 2003)
<i>Rhizomucor miehei</i>	Immobilized on an ion exchange resin	Sunflower oil	1:3 oil to methanol	79.1	(Deng et al., 2005; K. S. Moreira et al., 2020)
<i>Rhizomucor miehei</i>	Immobilized on magnetic nanoparticles	Babassu oil	1:6 oil to methanol	81.7	(K. da S. Moreira et al., 2020)

2.5.4 *Candida antarctica* Lipase B (CALB)

Another extensively utilized enzyme in biodiesel production is *Candida antarctica* lipase B (CALB), a highly robust and versatile biocatalyst renowned for its stability, selectivity and catalytic efficiency in a broad range of reaction media. CALB is most commonly available in commercially immobilized form under the trade name Novozym 435, where the enzyme is immobilized on a microporous acrylic resin to enhance reusability and operational stability. CALB has been the subject of intense biochemical research due to its industrial significance. The amino acid and genic sequence of CALB was first described by Uppenberg's research group revealing a protein consists of 317 amino acid residues with a molar mass of 33 kDa. The enzyme's catalytic triad is formed by Ser105, His224, and Asp187 (Höck et al., 2018), a configuration typical of serine hydrolases that enables the catalytic hydrolysis and transesterification of esters. What makes CALB is different from other lipases is its high enantioselectivity allowing it to preferentially catalyze reactions involving one enantiomer over another. This makes it not only ideal for biodiesel production but also for complex transformations such as kinetic resolutions, aminolysis, esterification, and transesterification in polar organic solvents. Its compatibility with non-aqueous environments makes CALB a powerful tool in synthetic organic chemistry.

Because of its good properties, numerous studies have been conducted to improve CALB's properties as a catalyst in other solutions, and one of the methods is immobilization. Immobilization can be done by various techniques such as non-covalent adsorption, deposition, single covalent attachment, multiple covalent attachments, entrapment in a polymeric gel in a membrane or capsule, cross-linking of an enzyme, and enzyme crystals (Kundys et al., 2018). Among these, immobilization on acrylic resin, as in Novozym 435 has gained prominence due to its mechanical strength and biocompatibility with hydrophobic substrates such as oils. The effectiveness of immobilized CALB as a biocatalyst in transesterification reactions is summarized in Table 2.13, which lists previous studies involving CALB for biodiesel synthesis.

Table 2.13
Biodiesel Synthesis Yield Using Immobilized *Candida antarctica* Lipase B as Biocatalyst

Lipase	Condition/ Method	Feedstock	Molar Ratio	Yield (%)	Reference
Novozym 435	Immobilization of Novozym 435 within mesoporous silica matrices; MPS synthesized via co-precipitation method at constant pH	Algal oil derived from <i>Nannochloropsis</i>	-	94.8	(Mirsalami & Mirsalami, 2024)
CALB	Sol-gel immobilization on functionalized magnetic nanoparticles	Waste Cooking Oil	1:4 oil to methanol	96	(Parandi et al., 2022)
CALB	Immobilized on modified polyporous magnetic cellulose beads Reaction time:2h	Yellow corn seed oil	1:1:6 oil to methanol	92.3	(Zhang et al., 2020)
CALB	Immobilized on	Residual	1:18 oil to	96.8	(K. S. Moreira et al., 2020)

(Novozym 435)	microporous acrylic resin Reaction time: 4h	babassu oil	ethanol		
CALB	Immobilized on magnetic nanoparticles Reaction time: 10 h Agitation speed: 150rpm	Palm fatty acid distillate (PFAD)	1:1.6 PFAD to methanol	82.74	(Gupta & Rathod, 2020)
CALB	Immobilized on silica nanoflowers Reaction time: 8.11h Temperature: 45.97°C	Waste oil	1:2.63 oil to methanol	98.5	(Wang et al., 2019)
CALB (Novozym 435)	Immobilized on macro-porous acrylic resin Reaction time: 12h Temperature: 39.5°C	Insect fat (Black soldier fly larvae)	1:14.64 oil to methyl acetate		(Nguyen et al., 2018)
CALB	Immobilized on methacrylic resin Reaction time: 6h	Waste animal fat	1:10 oil to methanol	87	(Pollardo et al., 2017)
CALB	Immobilized on macro-porous acrylic resin Reaction time: 18h	Corn oil	1:15 oil to methanol	91.4	(Gu et al., 2015)

2.6 Triacetin as By-product

Triacetin, also known as glyceryl triacetate or 1,2,3-triacetoxyp propane, is an organic compound with the chemical formula $C_3H_5(OCOCH_3)_3$. It is a colorless, oily liquid with multiple industrial applications and has been utilized for over 75 years across various sectors. Its versatility lies in its roles as a plasticizer, a solvent in cosmetic and

pharmaceutical formulations, a flavoring agent and adjuvant in food processing, and a binder in the manufacturing of solid rocket propellants (Quinn & Ziolkowski, 2015). Despite its relatively high market price, industrial applications typically demand high-purity triacetin ($\geq 99.9\%$), underscoring its value-added nature. Triacetin can be produced through the interesterification of triglycerides with methyl acetate, a pathway that distinguishes itself from traditional transesterification using methanol, which generates crude glycerol as a by-product. In contrast, the methyl acetate route produces triacetin, a compound with greater commercial value and broader usability than glycerol (Zainal et al., 2023). The molecular structure of triacetin is shown in Figure 2.7, illustrating its tri-ester configuration derived from glycerol and acetic acid.

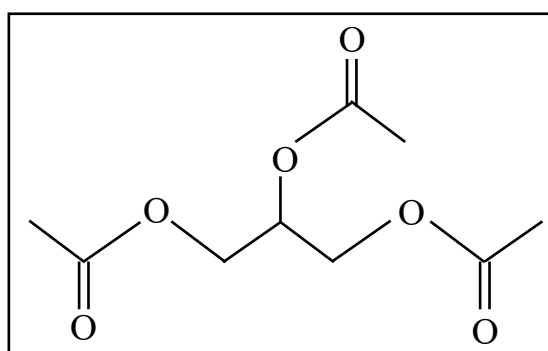


Figure 2.7 Structure of Triacetin

One of the key environmental advantages of triacetin is its non-toxic and biodegradable nature, with no significant environmental impact reported. Naturally occurring in small quantities in animal fats such as cod-liver oil and dairy products like butter, triacetin is also recognized as safe for use in food and pharmaceutical products. In biodiesel production via the interesterification route, triacetin formation offers an economic advantage. According to Casas et al., (2011), for every 100 kg of triglyceride oil, interesterification with methyl acetate yields approximately 100 kg of biodiesel and an additional 25 kg of triacetin, resulting in a total product yield of 125 kg. In comparison, conventional transesterification yields only biodiesel and crude glycerol, with the latter having limited market value and challenging purification requirements. If triacetin is incorporated into the final biodiesel formulation, it can contribute to increasing the total energy content of the biofuel blend. In fact, when all triglycerides are completely converted, triacetin can comprise up to 19.8% of the total weight of the biodiesel product (Casas et al., 2010). However, the increasing adoption of this process could potentially lead to market saturation, similar to what has been observed with glycerol in the past (Casas et al., 2010). Despite this, triacetin remains attractive due to

its miscibility with biodiesel and superior market price compared to crude glycerol. From both economic and environmental perspectives, the production of triacetin as a by-product in biodiesel synthesis presents a promising and sustainable pathway for enhancing the overall value and utility of renewable biofuels.

2.7 Ultrasonic Assisted-Enzymatic Transesterification

Ultrasonication refers to the application of sound waves with frequencies beyond the range of human hearing, typically above 20 kHz. These high-frequency vibrations carry mechanical energy, which can be harnessed to enhance various chemical and biochemical processes. In the context of enzymatic transesterification, lipases catalyze reactions at the interface between two immiscible phases, such as oil and alcohol or methyl acetate. This interfacial nature often results in mass transfer limitations, leading to a lag phase before the reaction becomes efficient. Ultrasonic energy mitigates this issue by promoting better phase contact and dispersion, thus minimizing the delay in reaction initiation and reducing overall reaction time. It also contributes to sustainable process development, aligning with the principles of green chemistry by reducing the need for harsh conditions or solvents (Bansode & Rathod, 2017).

Ultrasound equipment is generally categorized based on frequency and intensity. High frequencies with low power (1,000–10,000 Hz) are primarily used in medical applications, such as imaging and diagnostics. In contrast, low frequency with high power ultrasound (typically 20–1,000 Hz) is employed in chemical processing and materials science due to its ability to induce cavitation (Gusniah et al., 2019). Cavitation is the phenomenon where microscopic bubbles form, grow, and then collapse violently in a liquid medium. Figure 2.8 shows the energy was released as a result from the collapse bubbles in the solution. This collapse generates intense localized conditions of high pressure and temperature, even under ambient surroundings, and contributes significantly to enhanced mixing, mass transfer, and reaction rates.

The concept of ultrasonic cavitation was first linked to reaction enhancement in the mid-18th century when Leonhard Euler identified wave propagation principles that form the basis of ultrasonic interactions. Cavitation events can be categorized into several types which are optical, acoustic, hydrodynamic, and particle-induced where each defined by the mechanism through which bubbles are generated and collapse. In

ultrasonic-assisted reactors, these implosive bubble collapses act as microreactors that facilitate energy transfer, improve the interfacial area between reactants, and boost the availability of active enzyme sites (Bansode & Rathod, 2017; Parthasarathy et al., 2016; Talebian-Kiakalaieh et al., 2013). By employing ultrasonication, enzymatic transesterification processes become more efficient and environmentally favorable. The method offers a non-invasive means of intensifying biocatalytic activity while preserving enzyme integrity, especially when operated under optimized conditions. This synergy between ultrasound and enzymatic catalysis has shown promising potential in biodiesel production, particularly when dealing with viscous substrates like waste cooking oil and using green solvents such as methyl acetate.

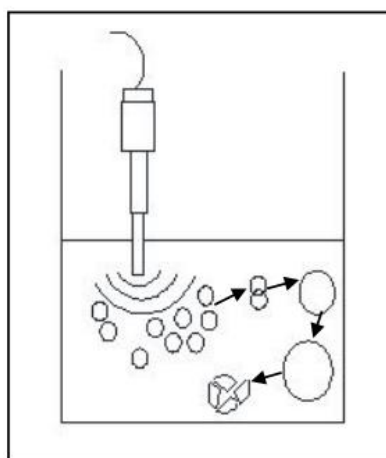


Figure 2.8 Bubble Collision Phenomenon During Cavitation
Source: Gusniah et al. (2019)

The main challenge in enzymatic transesterification for biodiesel production is the slow reaction rate, which directly affects biodiesel yield over time. This issue arises due to poor mixing of alcohol and oil, as they are immiscible, leading to weak interactions between the enzyme and the substrates (Bhangu et al., 2017). Additionally, the mild shock waves from collapsing bubbles enhance substrate transport towards the enzyme, improving interaction and eliminating mass transfer resistance, which often hinders enzymatic reactions. As a result, ultrasound technology significantly enhances mixing, accelerates reaction rates, and increases biodiesel yield in a shorter time.

Numerous studies have demonstrated the favorable impact of ultrasound on reaction kinetics in enzymatic biodiesel synthesis. For example, Adewale et al., (2016), reported that a biodiesel yield of 96.8% was achieved within just 20 minutes from waste lard using *Candida antarctica* lipase B under ultrasonic conditions compared to the 22-24 hours required using conventional approaches. Similarly, Bhangu et al., (2017),

observed that the use of *Candida rugosa* lipase type VII for transesterification of canola oil under ultrasound led to a 99% yield in only 90 minutes, drastically reducing the processing time from the usual 24 hours. S. Gupta et al., (2017), also have reported that about 98% biodiesel yield from neem oil catalyzed by engineered *Proteus vulgaris* lipase immobilized on imobeads within 30 minutes under ultrasonic-assisted conditions. A comparative study by Tupufia et al., (2013), highlighted the efficiency of ultrasound-assisted biodiesel production. Using Novozym 435, biodiesel from coconut oil achieved a 92% conversion in 3 hours with ultrasound, whereas conventional stirring at 350 rpm took 50 hours to reach 80% conversion. These findings consistently show that ultrasonic-assisted transesterification significantly reduces reaction time while increasing yield.

Research has consistently demonstrated that the use of ultrasound in enzymatic transesterification can drastically shorten reaction times while maintaining high biodiesel yields. In many instances, yields surpass 90%, with complete conversion occurring in under eight hours. This improved performance is largely attributed to the increased turbulence and convection within the reaction medium, generated by the collapse and movement of cavitation bubbles. These dynamic effects facilitate the formation of a fine emulsion between immiscible substrates, thereby improving contact between reactants and enhancing enzyme accessibility (Adewale et al., 2016). As a result, the application of ultrasonic waves not only accelerates the overall reaction rate but also promotes efficient mass transfer. Additionally, cavitation allows for optimal biodiesel conversion at lower enzyme concentrations, making the process more cost-effective and sustainable.

Ultrasound also reduces enzyme consumption. Bhangu et al., (2017) reported that only 0.68 wt% of lipase, based on oil weight, was required for enzymatic transesterification using ultrasonic assistance. This is because an enzyme concentration beyond the optimum level does not improve biodiesel yield. Instead, excessive enzyme tends to agglomerate, reducing the effective reaction area and lowering the overall yield. Similar findings were observed by Subhedar et al., (2015) and Subhedar & Gogate, (2016), where the required enzyme concentration in ultrasonic-assisted transesterification was reduced to 3% (w/v), compared to 6% (w/v) in conventional processes. Additionally, Jadhav & Gogate, 2014 demonstrated that biodiesel production from Nagchampa oil using 1000 mg of *Candida antarctica* lipase B achieved a high yield of 98% within 7.5 hours under ultrasonic conditions. Furthermore, most studies

indicate that the enzyme remains reusable, with less than a 10% loss in activity after several cycles, making ultrasonic-assisted enzymatic transesterification a more efficient and cost-effective approach. Therefore, the acoustic cavitation generated in ultrasonic-assisted transesterification significantly reduces the amount of enzyme required for the reaction. This effect is due to enhanced mass transfer and improved mixing of the reaction medium, which increases the interaction between the enzyme and the substrates. However, while ultrasound aids in the physical mixing of the liquid medium, the presence of lipase remains essential to catalyze the transesterification process effectively (Adewale et al., 2016; Kumar et al., 2011). Another key advantage of ultrasonic-assisted transesterification is its ability to reduce the required molar ratio of the acyl acceptor (such as methanol or methyl acetate) to oil, bringing it closer to the stoichiometric balance. This is because the cavitation effect improves substrate dispersion, promoting better contact between the reactants and allowing the reaction to reach equilibrium more efficiently. Consequently, ultrasonic-assisted enzymatic transesterification not only enhances reaction efficiency but also optimizes resource utilization, making it a promising approach for biodiesel production.

A study by Subhedar et al., (2015) highlighted the impact of ultrasonic-assisted transesterification on the molar ratio of alcohol to oil in biodiesel production. It was reported that the conventional method required a molar ratio of 1:4 (oil to methanol), while under ultrasonic-assisted conditions, a lower molar ratio of 1:3 was sufficient to achieve favorable biodiesel yields. Similarly, Adewale et al., (2016) recorded an optimal molar ratio of 1:4 under ultrasonic conditions, achieving a 98.2% biodiesel yield within just 20 minutes. Another study found that the same molar ratio produced an 84.5% biodiesel yield, further supporting the effectiveness of ultrasonic-assisted transesterification. The use of excess methanol in these reactions helps shift the equilibrium toward the formation of methyl esters, thereby enhancing biodiesel conversion. However, studies involving esters as acyl acceptors, such as methyl acetate, often require higher molar ratios even with ultrasonic assistance. For instance, Adewale et al., (2016) reported that a 1:12 molar ratio of oil to methyl acetate resulted in an 85.6% conversion after 6 hours, while Tavares et al., (2017), achieved a 98.25% yield under similar conditions. The need for excess methyl acetate arises due to its role in shifting the reaction equilibrium toward higher biodiesel conversion, emphasizing the importance of optimizing molar ratios based on the choice of acyl acceptor.

In overall, the production of biodiesel through enzymatic reactions can be significantly enhanced by using ultrasonic-assisted systems, as this approach reduces reaction time and lowers the required amounts of enzyme and acyl acceptor. This improvement is mainly due to cavitation, a phenomenon that occurs when ultrasonic waves generate microbubbles in the liquid. These acoustic bubbles undergo oscillation, growth, and rapid collapse, creating localized zones of high temperature and pressure. This intense micro-level activity improves mixing and mass transfer between the enzyme and reactants, leading to a more efficient transesterification process (Suslick et al., 2018). Cavitation happens when the pressure of a liquid drops too low, specifically below its vapor pressure. This often occurs at narrow points in a system, like an orifice where the fluid speeds up and pressure drops (Adewuyi, 2001). This energy dispersion enhances substrate mixing and improves contact between the substrates and the enzyme's active sites, facilitating a more efficient enzymatic reaction. Additionally, the uniform dispersion of ultrasonic waves ensures better substrate interaction and accelerates the achievement of equilibrium, promoting a faster reaction. By providing sufficient activation energy, ultrasonic-assisted transesterification improves reaction efficiency, increases biodiesel yield, and reduces overall production costs, particularly by lowering enzyme and reagent usage (Suslick et al., 2018).

Ultrasonic-assisted enzymatic transesterification offers several advantages over conventional methods, primarily by enabling hydrolysis and methanolysis to occur simultaneously rather than in a stepwise manner, leading to a more efficient reaction process (Adewale et al., 2015a). The intense mixing and cavitation effects generated by ultrasound enhance mass transfer, significantly increasing the reaction rate and reducing the time required to reach equilibrium (Tavares et al., 2017). Additionally, ultrasound improves enzyme reactivity, as studies have shown that sonicated enzymes exhibit higher activity compared to unsonicated ones due to better substrate interaction and exposure of active sites (Jadhav & Gogate, 2014). Another key benefit is the drastic reduction in separation time, where the by-product glycerol settles in less than 30 minutes compared to the conventional 5–10 hours, resulting in higher purity glycerol and more efficient phase separation (Kumar et al., 2011). Furthermore, ultrasonic-assisted systems enhance the thermostability of immobilized enzymes, with only partial enzyme inactivation observed within temperature variations of 30°C to 50°C, allowing for extended enzyme usability and multiple reaction (Gupta et al., 2017).

Despite its advantages, ultrasonic-assisted enzymatic transesterification has some limitations that can affect both biodiesel yield and enzyme activity. One major drawback is the formation of localized high-energy zones, or "hot spots," due to the collapse of cavitation bubbles as shown in Figure 2.9. This phenomenon generates extreme temperatures, free radicals, and intense shear forces from microstreaming and shockwaves, which can lead to enzyme inactivation and reduced catalytic efficiency (Bhangu et al., 2017). Additionally, since the acoustic energy from ultrasound is ultimately converted into heat, the reaction temperature tends to rise, which can further impact enzyme stability unless an effective cooling system is implemented (Kentish & Ashokkumar, 2011). To overcome this issue, one potential solution is to integrate ultrasonic-assisted transesterification with mechanical agitation. This approach helps distribute cavitation effects more uniformly across the reaction medium, preventing localized overheating and excessive enzyme deactivation while ensuring efficient mixing and mass transfer.

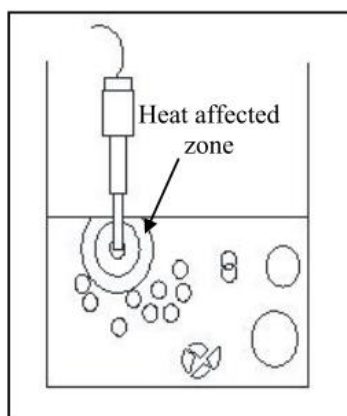


Figure 2.9 The Hot Spot of Energy Accumulation
Source: Gusniah et al. (2019)

Another factor that can decrease biodiesel yield in an ultrasonic-assisted enzymatic transesterification system is the excessive shear force generated at high power densities or intensities. When ultrasonic power is too high, it can lead to droplet coalescence and over-processing, which in turn reduces mass transfer efficiency and negatively impacts the reaction (Bhangu et al., 2017). Additionally, extreme cavitation at very high ultrasonic power can cause decoupling losses and cushioned cavity collapse, preventing effective energy transfer into the system and ultimately lowering biodiesel yield (Subhedar & Gogate, 2016). To optimize the process, ultrasonic power and temperature should be carefully controlled. High collapse temperatures shorten bubble lifetimes and increase localized heat accumulation, which can lead to enzyme

inactivation. By lowering ultrasonic power, bubble collapse temperatures are reduced, preventing excessive energy accumulation in hot spots. This approach ensures the formation of an appropriate volume of cavitation bubbles, generating controlled shear forces that enhance mass transfer without causing enzyme deactivation or excessive droplet coalescence (Brotchie et al., 2010). The summary of ultrasonic-assisted enzymatic transesterification is tabulated in Table 2.14.

Table 2.14
The Reaction Time and Biodiesel Conversion by Using Ultrasonic Assisted

Feedstock	Type of Immobilized Lipase	Reaction Conditions	Biodiesel Conversion (%)	References
Waste Cotton Seed Oil	<i>Thermomyces lanuginosus</i> lipase	1:4 oil to ethanol; Enzyme concentration: 3 wt%; Reaction Time: 3hr; Temp: 45°C; Ultrasonic frequency: 25kHz	96.5	(Yasvanthrajan et al., 2025)
Palm Oil	Liquid enzyme solution	1:6 oil to methanol; Reaction Time: 12hr; Temp:35°C; High frequency ultrasound-assisted	89	(Zheng et al., 2024)
Crude Palm Oil	<i>Candida antarctica</i> lipase A (CaLA)	1:9 oil to methyl acetate; Enzyme Loading: 1wt%; Reaction Time: 3hr; Temp: 45°C	80.6	(Zainal et al., 2023)
Ceiba Pentandra (<i>Kapok</i>) Oil	<i>Rhizopus oryzae</i>	1:6 oil to methanol; Enzyme Loading: 10wt%; Reaction Time: 4hr; Temp: 60°C	93	(Pasawan et al., 2022)
Jatropha Oil	<i>Pseudomonas cepacia</i>	1:3 oil to methanol; Enzyme Loading: 5wt%; Reaction Time: 6hr Temp: 45°C	76.55	(Veney et al., 2022)

Used Cooking Oil	<i>Oreochromis niloticus</i>	1:4 oil to methanol; Reaction Time: 3hr; Temp: 40°C; Ultrasonic frequency: 16kHz	97.59	(Patchimpet et al., 2021)
Waste Cooking Oil	<i>Thermomyces lanuginose</i>	1:3 oil to methanol Enzyme Loading: 5 wt%; Reaction Time: 6hr; Temp: 35°C	69.3	(Manab et al., 2021)
Blended non-edible Oils	Commercial immobilized <i>Thermomyces lanuginosus</i> lipase	1:7.64 oil to methanol; Enzyme Loading: 3.55wt%; Temp: 36°C	94	(Malani et al., 2019)
Canola Oil	<i>Candida rugosa</i> lipase Type VII30	1:5 oil to methanol; Enzyme Loading: 0.23wt%; Reaction Time: 1.5hr; Ultrasonic frequency: 20kHz	99	(Bhangu et al., 2017)
Macauba Oil Soybean Oil	Novozyme 435	1:9 oil to ethanol; Enzyme Loading: 20 wt%; Reaction Time: 0.5hr Temp: 40°C; Ultrasound power: 40W	75.2 88	(Santin et al., 2017)
Crambe Oil	Novozyme 435	1:12 oil to methyl acetate; Enzyme Loading: 20wt%; Reaction Time: 6hr	98.25	(Tavares et al., 2017)
Waste Lard	<i>Candida antarctica</i> lipase B	1:4 fat to methanol Enzyme Loading: 6wt% Reaction Time: 0.33hr (Under solvent free system)	96.8	(Adewale et al., 2016)
Waste	<i>Thermomyces</i>	1:9 oil to methyl	96.1	(Subhedar & Gogate, 2016)

Cooking Oil	<i>lanuginosus</i>	acetate; Enzyme Loading: 3wt%; Reaction time: 3hr; Temp:40°C		
Macauba	<i>Candida</i>	1:9 oil to methanol;	70	(Michelin et al., 2015)
Coconut Oil	<i>antarctica</i> (Novozym 435)	Enzyme Loading: 20 wt%; Temp: 65°C Reaction Time: 0.5hr		
Waste Tallow	<i>Candida</i> <i>antarctica</i> lipase B	1:12 oil to methyl acetate; Enzyme Loading: 20wt%; Reaction Time: 6hr	85.6	(Adewale et al., 2015a)
Waste Choice White Grease (CWG)	<i>Candida</i> <i>antarctica</i> lipase B	1:4 fat to methanol; Enzyme Loading: 6 wt%; Reaction Time: 0.33hr	98.2	(Adewale et al., 2015b)
Waste Cooking Oil	Novozym 435	1:6 oil to DMC Enzyme Loading: 10wt% Reaction Time: 4hr Temp: 60°C Agitation rate: 100rpm	86.61	(Jadhav & Gogate, 2014)
Coconut Oil	Novozym 435	1:3 oil to ethanol; Enzyme Loading: 1wt%; Reaction Time: 3hr; Temp: 50°C	92	(Tupufia et al., 2013)
Soybean Oil	<i>Candida</i> <i>antarctica</i> and <i>Rhizomucor</i> <i>miehei</i>	1:3 oil to ethanol; Enzyme Loading: 5wt%; Reaction Time: 4hr; Temp: 40°C	90	(Batistella et al., 2012)
<i>Jatropha</i> <i>Curcas</i> Oil	Immobilized lipase from <i>Enterobacte-</i>	1:4 oil to methanol; Enzyme Loading: 5wt%;	84.5	(Kumar et al., 2011)

<i>raerogenes</i> on	Reaction Time: 0.5hr
silica activated	
with	
ethanolamine by	
cross-linking	
with	
glutaraldehyde	

2.8 Kinetics Study of Enzymatic Transesterification

The kinetic study of biodiesel production via enzymatic transesterification has become an area of significant interest in recent years. A comprehensive understanding of the kinetics of enzymatic transesterification is crucial for optimizing the process. Kinetic models help to describe the reaction mechanisms and rates, providing insights into the factors influencing enzyme activity and stability. Experimental work combined with kinetic modelling allows researchers to predict the performance of enzymatic transesterification under various conditions, guiding the design and scaling up of biodiesel production processes. Recent studies have developed kinetic models that account for enzyme kinetics, substrate concentration, temperature, and other variables. These models aim to provide a realistic and detailed description of the transesterification process, facilitating the optimization of reaction conditions and the development of more efficient enzymatic biodiesel production methods.

2.8.1 Kinetic Modelling and Parameter Estimation

Enzyme-catalyzed transesterification has emerged as a favourable method for biodiesel production due to its environmental benefits and efficiency in processing diverse feedstocks. While acid and alkaline catalysts are commonly employed, the challenges associated with glycerol recovery, catalyst removal, and purification make enzymatic transesterification a preferable alternative. Enzyme-catalyzed reactions offer high specificity and milder operating conditions, which contribute to producing biodiesel of high quality. The kinetic parameters of enzyme-catalyzed reactions can often be described using the Michaelis-Menten equation, which characterizes the relationship between the reaction rate and substrate concentration:

$$v = \frac{v_{\max} [S]}{K_S + [S]} \quad (2.1)$$

The enzyme kinetic mechanism for single substrate as shown below:



Where,

V = The rate of reaction

$V_{\max}$ = The maximum rate of reaction

$[P]$ = The concentration of product accumulation

$[E]$ = The concentration of enzyme

$[S]$ = The concentration of substrate

However, while Michealis Menten equation provides a fundamental basis for understanding enzyme kinetics, it is primarily applicable to single-substrate enzymatic reactions. In the case of biodiesel synthesis via enzymatic transesterification, the reaction involves two substrates, typically a triglyceride (TG) and an alcohol (A), making the simple Michaelis Menten model insufficient to describe the full reaction dynamics.

To overcome this limitation, the enzyme (E) first binds to the first substrate, triglyceride (TG), to form an enzyme-substrate complex. This leads to the release of the first product, usually a partial glyceride or a fatty acid (F), and the formation of a modified enzyme intermediate (E'). The second substrate, alcohol (A), then binds to E', resulting in the formation of a second enzyme-substrate complex (E'A). This complex breaks down to release the second product (such as an ester or biodiesel molecule), and the enzyme is regenerated in its original form, ready to catalyze another reaction cycle. This stepwise mechanism is particularly relevant in enzymatic transesterification due to the nature of the substrates and the specificity of lipase enzymes. It also allows for the incorporation of kinetic parameters such as inhibition constants, which become significant when one of the substrates or products exerts inhibitory effects on the enzyme. Therefore, the Ping Pong Bi-Bi mechanism, illustrated in Figure 2.10 below, provides a more accurate representation of the reaction kinetics and plays a critical role in modelling, optimization and scale-up of enzymatic biodiesel production processes.

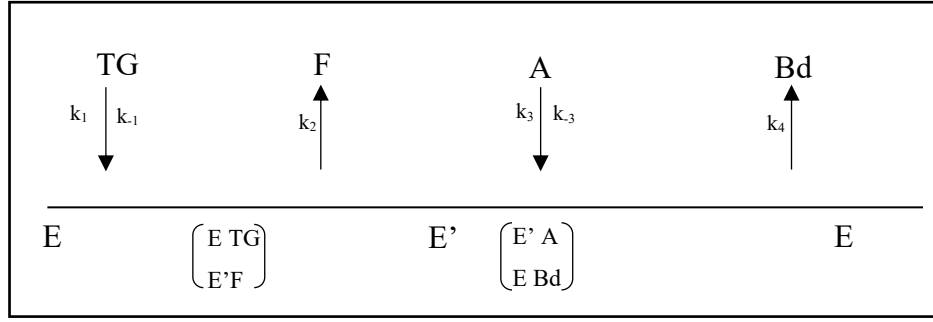


Figure 2.10 Lipase-Catalyzed Reaction Mechanism Following Ping Pong Bi-Bi Pathway Based on Cleland Nomenclature

The Marangoni approach can be used in order to simplify the model enzyme kinetic equation with Ping Pong Bi-Bi mechanism.

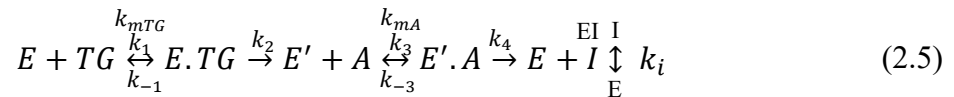
$$\frac{v}{v_{max}} = \frac{[TG][A]}{\frac{k_4}{k_2} k_{mTG}[A]} + k_{mA}[TG] + [TG][A](1 + (k_4 + k_2)) \quad (2.3)$$

When k_4 is the rate limiting constant, the equation will be;

$$\frac{v}{v_{max}} = \frac{[TG][A]}{\alpha k_{mTG}[A]} + k_{mA}[TG] + [TG][A] \quad (2.4)$$

Where $\alpha = k_4/k_2 = 1$

The inhibition constant is added when inhibition of substrate exists and called as competitive reversible inhibition. Therefore, the general schemes in steady state becomes:



The rate of reaction with inhibition is based on Michaelis Menten kinetic equation:

$$v = v_{max} \frac{s}{k_s \left(1 + \frac{[A]}{k_{iA}}\right)} + S \quad (2.6)$$

Where I is the inhibitor (methyl acetate). After applying the inhibition term and combining Ping Pong mechanism of equation 2.3, the equation becomes:

$$v = \frac{v_{max} [TG][A]}{k_{mTG}[A] \left(1 + \frac{[A]}{k_{iA}}\right) + k_{mA}[TG] + [TG][A]} \quad (2.7)$$

Equation 2.7 can be used for estimating of initial rate of transesterification of triglyceride with different types of acyl acceptor. Table 2.15 summarizes previous

kinetic modelling studies on enzyme-catalyzed transesterification based on Ping Pong Bi-Bi mechanism.

Table 2.15
Summary of Kinetic Modelling of Enzyme-catalyzed Transesterification Based on Ping Pong Bi-Bi Mechanism

Oil Source and Catalyst	Reaction Conditions	Kinetic Model Parameters	References
Kapok seed oil Catalyst: Lipozym TL IM	Horizontal water bath shaker Temp: 40°C	$V_{\max} = 0.000468 \text{ Ms}^{-1}$ $K_{\text{mTG}} = 0.000107\text{M}$ $K_{\text{mA}} = 0.00176\text{M}$	(Gunawan et al., 2017)
Jatropha oil Catalyst: Lipozyme RM IM (1g)	Reactor: Circulated batch packed bed reactor (CBPBR) Temperature: 45°C	$V_{\max} = 0.0000037 \text{ Ms}^{-1}$ $K_{\text{mTG}} = 0.0067 \text{ M}$ $K_{\text{tA}} = 2.212\text{M}$ $K_{\text{mA}} = 0.042\text{M}$	(Veney et al., 2014)
Waste Frying Oil Catalyst: Novozym 435	Semi-continuous reactor Temperature: 44.5°C	$V_{\max} = 0.0003 \text{ Ms}^{-1}$ $K_{\text{mTG}} = 397\text{M}$ $K_{\text{tA}} = 1815\text{M}$ $K_{\text{mA}} = 1030 \text{ M}$	(Azócar et al., 2014)
Waste Cooking Palm Oil Catalyst: Novozym 435 (0.4g)	Batch shaker	$V_{\max} = 0.0156 \text{ Ms}^{-1}$ $K_{\text{mTG}} = 2.61\text{M}$ $K_{\text{iA}} = 10.25\text{M}$ $K_{\text{mA}} = 1.6\text{M}$	(Halim & Harun Kamaruddin, 2008)
Waste Cooking Oil Novozym 435 (0.5g)	Batch shaker Temperature: 40°C	$V_{\max} = 0.03167 \text{ Ms}^{-1}$ $K_{\text{mTG}} = 1\text{M}$ $K_{\text{tA}} = 0.0455\text{M}$ $K_{\text{mA}} = 16\text{M}$	(Xu et al., 2005)
Sunflower oil Catalyst: <i>Rhizomucor miehei</i>	Lipase is immobilized on ion- exchange resins	$V_{\max} = 0.0069 \text{ Ms}^{-1}$ $K_{\text{mTG}} = 0.13\text{M}$ $K_{\text{iA}} = 0.000098\text{M}$ $K_{\text{mA}} = 1.9\text{M}$	(Al-Zuhair, 2005)
Sunflower oil Catalyst: <i>Thermomyces lanuginosa</i>	Lipase is immobilized on silica gel	$V_{\max} = 0.00328 \text{ Ms}^{-1}$ $K_{\text{mTG}} = 0.13\text{M}$ $K_{\text{iA}} = 0.000482\text{M}$ $K_{\text{mA}} = 0.9\text{M}$	(Al-Zuhair, 2005)
Sunflower oil Catalyst: Lipozyme RM IM (0.01g)	Glass tube stirrer batch	$V_{\max} = 0.0000042 \text{ Ms}^{-1}$ $K_{\text{mTG}} = 0.0053\text{M}$ $K_{\text{tA}} = 0.055\text{M}$ $K_{\text{mA}} = 0.013\text{M}$	(Dossat et al., 2002)

Castor oil	-	$V_{\max} = 0.0002 \text{ Ms}^{-1}$	(Krishna & Karanth, 2001)
Catalyst:		$K_{mTG} = 3030\text{M}$	
Novozym 435		$K_{mA} = 3060\text{M}$	
		$K_{iTG} = 1.05\text{M}$	
		$K_{iA} = 6.55\text{M}$	

Kinetic data obtained experimentally can be modelled to support scale-up analysis and to evaluate the influence of reaction parameters on enzymatic transesterification processes. However, studies reporting kinetic modelling of biodiesel production via transesterification with methyl acetate, particularly those incorporating substrate inhibition effects, remain limited in the literature. The Ping-Pong Bi-Bi model has been reported as an appropriate framework for describing lipase-catalysed transesterification reactions involving two substrates. Kinetic parameters are commonly validated using numerical modelling tools such as Polymath.

2.9 Biodiesel Fuel in Diesel Engine and Its Emission

The global reliance on petroleum diesel as a primary transportation fuel has led to significant environmental challenges, particularly due to its contribution to air pollution and greenhouse gas emissions (Liu et al., 2023). Diesel engines are known to emit pollutants such as nitrogen oxides (NO_x), carbon monoxide (CO), carbon dioxide (CO₂), unburned hydrocarbons (HCs), particulate matter (PM), and sulfur oxides (SO_x) (Wahaab et al., 2022). These emissions are harmful not only to the environment but also to human health, causing respiratory and cardiovascular issues and contributing to smog formation and climate change (Jarkoni et al., 2022). In response to these issues, biodiesel has emerged as a renewable and cleaner-burning alternative to conventional diesel fuel (Rahman Adib et al., 2024).

Biodiesel is typically produced through a chemical process known as transesterification, in which triglycerides from vegetable oils or animal fats react with short-chain alcohols (commonly methanol or ethanol) in the presence of a catalyst, yielding fatty acid methyl esters (FAMEs) and glycerol (Malla et al., 2024). These FAMEs are what constitute biodiesel. Unlike fossil-based fuels, biodiesel is biodegradable, non-toxic, and derived from renewable sources, making it an environmentally friendly alternative (Karmarkar, 2023). Moreover, it possesses

favorable fuel properties such as high lubricity, good cetane number and the presence of inherent oxygen content, which promotes more complete combustion (Malla et al., 2024). The combustion of biodiesel is cleaner due to its higher oxygen content (typically around 10–12%), which facilitates better oxidation of hydrocarbons during engine operation. As a result, engines running on biodiesel emit lower amounts of CO, CO₂, HC, and PM, making it a valuable tool in reducing the environmental footprint of diesel-powered transportation and industrial machinery.

One of the most attractive aspects of biodiesel is that it can be used in existing compression ignition (CI) engines with minimal or no modifications, either as pure biodiesel (B100) or in blends with conventional diesel (Rajendran et al., 2023), such as B5 (5% biodiesel), B10 (10% biodiesel), B20 (20% biodiesel), and so on. Numerous studies have shown that these blends retain similar performance characteristics to petroleum diesel, such as engine power output and fuel consumption, while offering significant reductions in tailpipe emissions (Azmeer et al., 2025). However, one area of concern is the tendency for some biodiesel blends to increase NO_x emissions (Rahman Adib et al., 2024), which are precursors to ground-level ozone and smog (Chauhan & Shukla, 2011). This effect varies depending on the feedstock used, combustion conditions, and engine calibration (Alahmer et al., 2022), and it continues to be an area of active research.

The growing interest in biodiesel is also driven by national and international policies aimed at reducing greenhouse gas emissions and increasing the use of renewable energy. For instance, many countries have adopted biodiesel mandates that require a certain percentage of biodiesel in diesel fuel sold commercially. In Malaysia, for example, the government has progressively implemented biodiesel blending policies, such as B7 and B10, with plans to increase the blend ratio in support of environmental goals and palm oil industry development. From an engine emissions perspective, biodiesel combustion generally results in lower carbon monoxide (CO) emissions due to improved oxidation, reduced unburned hydrocarbons owing to its oxygenated nature, decreased particulate matter particularly in terms of soot formation, and a substantial reduction in sulfur oxides since biodiesel contains negligible sulfur. However, the effect on nitrogen oxides (NO_x) emissions is variable and may show a slight increase depending on combustion temperature and the specific composition of the biodiesel.

2.9.1 NO_x Emission

Nitrogen oxides emissions are one of the most critical pollutants associated with diesel engine combustion. These emissions contribute significantly to environmental problems such as photochemical smog, acid rain, and ground-level ozone formation, as well as respiratory health issues (Chauhan & Shukla, 2011). While biodiesel is recognized for its cleaner combustion characteristics and ability to reduce pollutants like carbon monoxide, hydrocarbons, and particulate matter, its influence on NO_x emissions is more complex and has been widely debated in the scientific community. Some studies have reported increases in NO_x with biodiesel use, whereas others have observed variable or reduced NO_x emissions depending on engine type, fuel blend, and operating conditions

Numerous experimental studies have indicated that using biodiesel in diesel engines may result in an increase in NO_x emissions compared to conventional diesel (Alahmer et al., 2022). This tendency is primarily linked to the higher oxygen content present in biodiesel. The additional oxygen enhances combustion efficiency and promotes more complete fuel oxidation (Siddeg et al., 2022). However, it also increases the peak combustion temperature within the engine cylinder, which in turn accelerates thermal NO_x formation (Xue et al., 2011).

For instance, Tanwar et al., (2026), investigated biodiesel from edible oil refinery waste (Transesterified Acidic Oil, TAO) in a single-cylinder diesel engine. NO_x emissions were blend-dependent: while a low-concentration blend (D–TAO5) slightly reduced NO_x to 1000 ppm compared with 1010 ppm for diesel, higher blends such as D–TAO10 reached 1190 ppm, highlighting the tendency of oxygen-rich biodiesel to increase thermal NO_x formation at higher concentrations. Intermediate blends (D–TAO15 and D–TAO20) showed moderate NO_x levels of 1052 and 1090 ppm, respectively. Similarly, Loh et al., (2026), reported that enzymatic biodiesel from sludge palm oil, both crude (CEB) and partially purified (PPEB), exhibited higher NO_x emissions than conventional diesel, mainly due to elevated combustion temperatures, oxygen content, and physical properties such as viscosity and cetane number that affected injection timing and combustion dynamics.

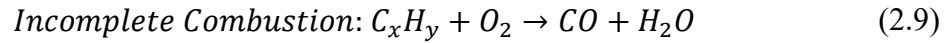
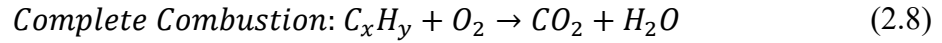
Other studies confirm the link between biodiesel's oxygen content, combustion temperature, and NO_x formation. Narayanan et al., (2025), observed that *Momordica charantia* biodiesel–diesel blends (B10–B30) produced higher NO_x emissions at

elevated loads, with advanced combustion phasing, shorter ignition delay, and increased premixed heat release further contributing to higher NO_x, particularly for B30. T. Li & Liang, (2024), reported similar trends for *Annona reticulata* L. oil biodiesel blends, where elevated in-cylinder temperatures and oxygen availability at higher loads promoted NO_x formation. Sakkampang et al., (2023), found that crude and distilled Yang-Na biodiesel blends also exhibited increasing NO_x emissions with higher blending ratios, with crude biodiesel producing the highest NO_x due to enhanced heat release and elevated combustion temperatures.

Despite this common trend, several studies have reported reductions in NO_x emissions with specific fuel modifications or additive strategies. Suteerapongpun et al., (2026), showed that ethanol blending with standard B7 diesel could reduce NO_x at high loads, with the B7E10 blend producing the lowest NO_x, attributed to ethanol's cooling effect via latent heat of vaporization, which lowered peak in-cylinder temperatures. Mehta et al., (2025), demonstrated that adding Diethyl Carbonate (DEC) to Karanja biodiesel (B20) progressively reduced NO_x emissions, with a 10% DEC blend achieving 6% lower NO_x than pure diesel. The reduction was due to DEC's high latent heat and cetane number, which moderated peak temperatures and shortened ignition delay, producing more controlled combustion. Sonachalam et al., (2024), reported that an optimized blend of 20% biodiesel, 10% ethanol, and 30 ppm multi-walled carbon nanotubes (MWCNT) reduced NO_x by up to 35%, attributed to improved combustion efficiency and enhanced fuel–air mixing. Ayazi et al., (2023), also observed reductions in NO emissions (11–14%) for ternary blends of biodiesel, bioethanol, and diesel (B10E10D80, B10E15D75, B10E20D70) due to the cooling effect of bioethanol suppressing thermal NO_x formation.

2.9.2 CO Emission

Carbon monoxide (CO) is a toxic gas that forms as a result of incomplete fuel combustion in internal combustion engines. In diesel engines, the generation of CO is typically lower than in gasoline engines due to higher combustion efficiency. However, the nature of the fuel plays a significant role in determining the extent of CO emissions. Chemically, CO is produced when carbon in the fuel is only partially oxidized, as represented in the simplified combustion reactions in Equation (2.9):



Biodiesel generally leads to a reduction in CO emissions compared to conventional diesel due to its higher oxygen content, which enhances the oxidation process and favors the conversion of carbon monoxide (CO) to carbon dioxide (CO₂).

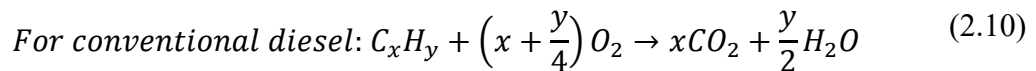
Many studies have reported that the use of biodiesel and its blends generally lead to a reduction in CO emissions when compared to conventional diesel. For example, Siam et al., (2026), reported substantial reductions in CO emissions using tobacco seed biodiesel blends, with B15 and B5 achieving average CO reductions of 76.75% and 63.79%, respectively, compared to diesel. The reduction was attributed to improved combustion efficiency resulting from higher oxygen content and cetane number. The addition of Al₂O₃ and CuO nanoparticles further enhanced fuel atomization and ignition characteristics, leading to additional CO reduction. Similar trends were observed by Y. Liu et al., (2026), who investigated castor oil biodiesel (C20) blended with hydrogen and TiO₂ nanoparticles, reporting 7–28% lower CO emissions across all engine loads compared to diesel. Likewise, Tec-Caamal et al., (2025) found that waste cooking oil–karanja biodiesel blends (WKB20) produced consistently lower CO emissions than diesel, with reductions of up to 35% when CeO₂ nanoparticles and HHO gas were introduced, owing to improved air–fuel mixing, higher in-cylinder temperatures, and supplementary oxygen availability. Reductions in CO emissions with increasing biodiesel fraction were also reported for *Sterculia foetida* biodiesel by Devarajan & D, (2024), and for microalgae-derived biodiesel by Tizvir et al., (2023) with the latter reporting up to 50% CO reduction for a 20% biodiesel blend. Vehicle-based and transient-condition studies further support these findings. Y. Zhang et al., (2022), demonstrated that waste cooking oil biodiesel blends (B10–B20) consistently reduced CO emissions in an urban diesel bus under idle, acceleration, and deceleration modes, with reductions becoming more pronounced at higher blend ratios. The presence of after-treatment systems, such as diesel oxidation catalysts and catalyzed diesel particulate filters, further amplified CO mitigation, achieving reductions of up to 58%.

Despite these generally favorable outcomes, several studies have reported increased CO emissions under specific operating conditions or fuel formulations. Ge et al., (2022) observed higher CO emissions when crude palm oil–diesel blends were used

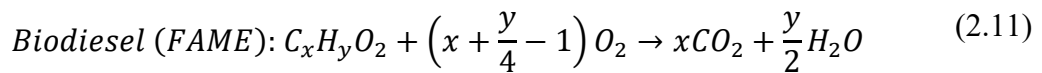
under idling conditions, with increases attributed to the high viscosity and density of crude palm oil, which impaired atomization and led to locally fuel-rich zones. Similarly, Yesilyurt & Aydin, (2020), reported mixed effects when diethyl ether was added to cottonseed biodiesel blends: low concentrations reduced CO emissions, whereas higher concentrations increased CO formation due to charge cooling and delayed ignition. In addition, Sidibe et al., (2020), found that CO emissions from *Jatropha curcas* vegetable oil and its blends remained comparable to diesel at low and medium loads but increased significantly beyond 80% engine load. Diesel consistently exhibited the lowest CO emissions, and the application of exhaust gas recirculation (EGR) was found to exacerbate CO formation due to reduced oxygen availability and poorer combustion quality.

2.9.3 CO₂ Emission

Carbon dioxide (CO₂) is one of the most significant greenhouse gases contributing to global warming and climate change (Aljaafari et al., 2022; Hoa et al., 2024). Its mitigation is a central goal in transitioning toward sustainable energy systems, particularly in the transport sector. In internal combustion engines, CO₂ is primarily generated through the complete oxidation of carbon present in the fuel. For conventional diesel fuel, which can be approximated by the general hydrocarbon formula C_nH_m, the combustion reaction may be expressed as:



In contrast, biodiesel, typically composed of fatty acid methyl esters (FAME), has a general molecular formula of C_xH_yO₂, and its combustion can be represented as:



According to Kamil et al., (2019), equation (2.4) indicates that the theoretical amount of CO₂ formed during complete combustion is directly proportional to the number of carbon atoms in the fuel. In the case of fossil diesel, which is commonly approximated by the empirical formula C₁₂H₂₃, the combustion of n moles of fuel produces 12n moles of CO₂ under stoichiometric conditions. These equations highlight that the carbon in biodiesel is already partially oxidized due to the oxygenated structure of fatty acid methyl esters, which can improve combustion efficiency (Kamil et al.,

2019).

Beyond combustion chemistry, the environmental advantage of biodiesel lies in its carbon origin. The CO₂ released during biodiesel combustion is generally regarded as part of a short-term biogenic carbon cycle, as it is largely offset by the CO₂ absorbed by biomass feedstocks during photosynthesis (Xue et al., 2011). This contrasts with fossil diesel, where carbon released during combustion originates from geological reservoirs and contributes directly to net atmospheric CO₂ accumulation. As a result, biodiesel offers a pathway for reducing net greenhouse gas emissions without requiring fundamental changes to existing compression ignition engines.

Life cycle assessment (LCA) studies have consistently demonstrated the carbon mitigation potential of biodiesel when emissions from feedstock production, fuel processing, transportation, and combustion are considered. Reported reductions in overall CO₂ emissions of up to 80% relative to petroleum diesel have been observed, with the most pronounced benefits associated with biodiesel derived from waste-based feedstocks or non-food crops (Carraretto et al., 2004; Lapuerta et al., 2008). The utilization of such feedstocks avoids emissions linked to agricultural cultivation and land-use change, thereby further enhancing the sustainability profile of biodiesel.

Experimental investigations of engine exhaust emissions generally indicate that biodiesel and biodiesel–diesel blends produce comparable or reduced CO₂ emissions relative to conventional diesel, although the magnitude of reduction varies with blend ratio and operating conditions. Sekhar & Gupta, (2025), reported that rapeseed biodiesel–diesel blends reduced CO₂ emissions, with the R20 blend achieving a maximum reduction of 6.7%, attributed to biodiesel’s lower carbon content and higher oxygen availability. Similarly, Nabi et al., (2022) observed a maximum CO₂ reduction of 17.86% for a waste tire oil–biodiesel–diesel blend (30T10B), which was linked to its lower carbon-to-hydrogen (C/H) ratio compared to diesel. Reductions in CO₂ emissions have also been reported for neem oil biodiesel blends (Mathiyazhagan et al., 2022) and biodiesel–diesel blends derived from microalgae and biomass oils under optimized operating conditions (Karpanai Selvan et al., 2022).

However, not all studies report a reduction in tailpipe CO₂ emissions. Some investigations have documented marginal increases in CO₂ when biodiesel blends are used. For instance, V et al., (2025) reported a slight increase of approximately 0.42% in CO₂ emissions for a 20% algae biodiesel blend at full engine load, attributed to enhanced combustion resulting from the fuel’s oxygen-rich composition. Similar trends

were reported by Rahman Adib et al., (2024) and from Wu et al., (2019), who associated increased CO₂ emissions with higher fuel consumption, elevated exhaust gas temperatures, and improved oxidation of carbon species. These findings suggest that enhanced combustion efficiency and oxygen availability in biodiesel may, under certain conditions, promote greater conversion of CO to CO₂.

It is therefore important to note that while tailpipe CO₂ measurements may vary depending on engine load, operating conditions, and fuel composition, the overall environmental impact of biodiesel should be assessed from a life cycle perspective. Even when tailpipe CO₂ emissions appear higher, the net carbon contribution to the atmosphere remains lower for biodiesel than for fossil diesel, due to the carbon-neutral nature of the feedstock. The comparative result of each investigation related to emission profiles of various biodiesel/diesel blends across different engine types is documented in Table 2.16 below:

Table 2.16
Emission Profile of Various Biodiesel/Diesel Blends Across Different Engine Types

Fuel Type	Methodology	Key Findings	References
B10, B20, B30 & B40	Single-cylinder CI engine; dynamometer testing; exhaust gas analysis	CO ↓ ~33%; HC ↓ ~23%; NO _x ↑ (peak at B30); B20 optimum	(Azmeer et al., 2025)
Algae biodiesel blends (B0, B10, B20 & B30)	Single-cylinder CI engine; variable compression ratio; load variation; ASTM fuel testing	B10 vs diesel: BTE ↑ 1.7%, BSFC ↓ 12.7%, UHC ↓ 12%; NO _x ↑ 2.4%, CO ≈ CO ₂ ; Overall trend (↑ blend ratio): CO ₂ ↓, NO _x ↓, CO ↑ slightly, UHC ↑ slightly	(Alzubaidi et al., 2024)
B20 Cottonseed biodiesel + CeO ₂ & Al ₂ O ₃ (20-60 ppm each)	CI engine testing (CT110); 1800-2800 rpm; 80% load; GC-MS; sonication; emission analysis	Best blend (B20 – Al60Ce60): CO ↓ 30.1%, HC ↓ 65.8%, NO _x ↓, 55.4%, BSFC ↓, BT ↑, BP ↑	(Mangesha et al., 2025)
CB20 castor biodiesel + CeO ₂ / Al ₂ O ₃ nanoparticles	Single-cylinder CI engine; constant load; speed variation (1250–3200 rpm); nano-fuel	Best blends: CB20A50C50 & CB20A40C40 → BP ↑ 6.14% & 1.25%; BSFC ↓ 3.21–8.29%; BTE ↑ 20.38% (vs CB20);	(Tesfaye Lamore et al., 2023)

(80–100 ppm; single & hybrid)	preparation via sonication; emission analysis	Emissions: HC ↓ 29.5%, CO ↓ 38.3% (CB20C100); NOx ↓ 10.2% (CB20A50C50)	
Waste cooking-oil biodiesel blends (e.g. B10)	Urban bus testing; real driving conditions; speed & acceleration variation; exhaust after-treatment (DOC–CDPF)	Biodiesel effect: CO ↓, THC ↓, PN ↓, PM ↓; CO ₂ ↑, NOx ↑ (↑ with blend ratio, speed & acceleration); After-treatment: CO ↓ 58.0%, THC ↓ 53.1% (B10); PN ↓ >99%, PM ↓ 94% (fuel-independent)	(Zhang et al., 2022)
Tamarind seed biodiesel blends (TB5, TB10, TB15)	Single-cylinder CI engine; constant speed (1500 rpm); CR = 18; performance & emission analysis	TB blends vs diesel: CO ↓ (max 27% at TB10), HC ↓ (max 25% at TB5), smoke ↓ 15% (TB10), NOx ↓ (max 19.36% at TB15); BSFC ↑; Optimum: TB10	(Nair & Murthy, 2021)
Palm biodiesel blend (B30) + TiO ₂ nanoparticles (25 ppm) with EGR	CI engine testing; load variation; EGR application; nano-additive blending; emission & performance analysis	PBN–EGR vs PB–EGR: BSFC ↓, HC ↓, CO ↓; smoke ↓ (high load); NOx ≈ (on par); EGT ↑; cylinder pressure ↓ slightly; heat release rate ↑	(Venu et al., 2019)
Mahua biodiesel + dimethyl carbonate additive	Single-cylinder water-cooled CI engine; load variation; additive percentage variation; performance & emission analysis	↑ additive %: BTE ↑, BP ↑, BSFC ↓, EGT ↓; CO ↓, HC ↓, smoke ↓, NOx ↓	(Nayak & Pattanaik, 2014)
Waste-cooking oil biodiesel blends (B10, B20, B30)	Diesel engine testing; load variation (0–100%); performance & emission analysis	Vs diesel: BSFC ↑, BTE ↓, EGT ↑, CO ₂ ↓, NOx ↑, CO ↓, HC ↓, smoke ↓; trend: ↑ biodiesel fraction: BSFC ↑, CO & HC ↓, NOx ↑	(Abed et al., 2018)
Cotton, Jatropha & Neem biodiesel blends (B5–B30; binary & multi-blends)	4-cylinder stationary diesel engine; speed variation (1500–2500 rpm); exhaust gas analysis (IMR 1400)	B20C vs diesel: CO ↓, CO ₂ ↓, NOx ↓; EGT ↓; combustion efficiency ↑; SO ₂ ↑ (negligible); Trend: binary blends outperform multi-blends in emission reduction	(Kaisan et al., 2017)
Biodiesel	Diesel engine testing;	B20 vs diesel: HC ↓ ~20%, CO	(Ghenai, 2011)

blends from WCO (2 sources), peanut & coconut oils (B5–B20)	speed variation (1250–3550 rpm); dynamometer & gas analyzer measurements; repeated trials	↓ ~12%; NO _x ↑ ~2%; engine power ≈ (<2% change); CO ₂ ≈; Trend: ↑ biodiesel fraction → HC & CO ↓ consistently across all feedstocks	
Waste cooking oil methyl ester (WCO-ME)	DI diesel engine; constant speed (1500 rpm); full-load testing; performance, emission & combustion analysis	vs diesel: BTE ↓ 6%, BSEC ↑ 25.9%; CO ↓ 43.3%, HC ↓ 52.7%, NO ↓ 23%, smoke ↓ 15.5%; HRR ↓; ignition delay ↓; combustion duration ↑	(Kannan et al., 2011)

↑ = increment; ↓ = decrement

Enzymatic biodiesel production has attracted increasing attention as a sustainable alternative to conventional chemical transesterification due to its mild operating conditions, high product purity, and environmental compatibility (Wong et al., 2023). Immobilized lipases such as *Candida antarctica* lipase B (CALB) and *Rhizomucor miehei* lipase have been widely reported to offer superior stability and reusability (Marín-Suárez et al., 2019), while methyl acetate has emerged as a promising acyl acceptor due to its non-inhibitory nature toward lipases and its ability to generate triacetin as a value-added by-product (Zainal et al., 2023). Nevertheless, most reported studies focus primarily on biodiesel yield and conversion efficiency, with limited attention given to detailed kinetic behavior under intensified reaction conditions.

In particular, enzyme kinetic studies for lipase-catalyzed transesterification are predominantly conducted under conventional mechanical stirring, despite evidence that ultrasonic irradiation can significantly enhance mass transfer and reaction rates (Yasvanthrajan et al., 2025). The influence of ultrasonic cavitation on enzyme–substrate interactions and reaction pathways has not been sufficiently quantified using rigorous kinetic models. As a result, there is a lack of reliable kinetic parameters that accurately describe enzymatic biodiesel synthesis under ultrasonic-assisted systems.

Furthermore, the transesterification of triglycerides with methyl acetate constitutes a two-substrate reaction (Ruzich & Bassi, 2011). However, many existing kinetic analyses oversimplify the system by assuming pseudo–single-substrate behavior or neglecting substrate inhibition effects (Ezzati et al., 2021). Comprehensive kinetic modeling that accounts for both triglyceride and acyl acceptor concentrations, including the determination of key parameters such as maximum reaction rate ($V_{\max}$), Michaelis-

menten constants for triglyceride and acyl acceptor, (K_{mTG}) and (K_{mA}) values, and inhibition constant (K_i) based on appropriate mechanisms such as the Ping Pong Bi-Bi model, remains scarce (Xu et al., 2005). This limitation is particularly evident for immobilized CALB operating under ultrasonic conditions.

Although optimum substrate molar ratios for maximizing biodiesel yield have been reported, many kinetic studies are conducted over excessively wide and non-optimal concentration ranges. Such approaches may mask the intrinsic catalytic behaviour of the enzyme and reduce the reliability of kinetic parameters. Consequently, there is a lack of kinetic investigations that focus on substrate concentration variations around experimentally determined optimum molar ratios, such as the 1:12 waste cooking oil to methyl acetate ratio where CALB demonstrates maximum biodiesel yield

In addition, while numerous studies evaluate the combustion and emission characteristics of biodiesel fuels, few have established a direct relationship between enzymatic reaction kinetics, resulting fatty acid methyl ester composition, and exhaust emissions. The impact of biodiesel produced via enzymatic transesterification, particularly from waste cooking oil using methyl acetate on carbon monoxide, carbon dioxide, and nitrogen oxides emissions remains insufficiently explored, limiting the understanding of how upstream reaction pathways influence downstream engine performance.

Overall, existing studies rarely integrate waste-derived feedstocks, immobilized enzymatic catalysis, ultrasonic-assisted processing, detailed kinetic modeling, and emission analysis within a single framework. Consequently, a comprehensive investigation that simultaneously addresses enzymatic reaction kinetics, inhibition behavior, and emission characteristics under an ultrasonic-assisted system using waste cooking oil is still lacking. Addressing these gaps is essential for advancing the practical and environmental viability of enzymatic biodiesel production.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Summary of Methodology

The methodology begins with the enzymatic transesterification of waste cooking oil (WCO) and methyl acetate in an ultrasonic system, using immobilized lipases to produce biodiesel. Two immobilized lipases, *Rhizomucor miehei* (RMIM) and *Candida antarctica* lipase B (CALB), were compared across a range of molar ratios from 1:3 to 1:12 (WCO:methyl acetate) to evaluate their effects on biodiesel yield. The produced biodiesel was characterized by using GC/MS to analyze its FAME. In addition, to prove that triacetin was formed in biodiesel production, the biodiesel and pure WCO were analyzed using FTIR in order to determine their functional groups. The study incorporates the Ping-Pong Bi-Bi mechanism with competitive inhibition, which describes the interaction between the two substrates, WCO and methyl acetate, with the enzyme, and accounts for product formation and release dynamics. A detailed kinetic study was conducted to evaluate kinetic parameters such as V_{max} , K_{mTG} , K_{mA} and K_{iA} . To ensure the reliability of the proposed kinetic model, experimental data was validated using non-linear regression analysis in Polymath 6.10 software, confirming the agreement between experimental outcomes and proposed kinetic model. Finally, the biodiesel was blended with diesel and tested for engine performance and emissions focusing on the duration of engine to operate and emissions of pollutants such as NO_x, CO and CO₂. The overall process flow diagram for the overall work of the research is as shown in Figure 3.1.

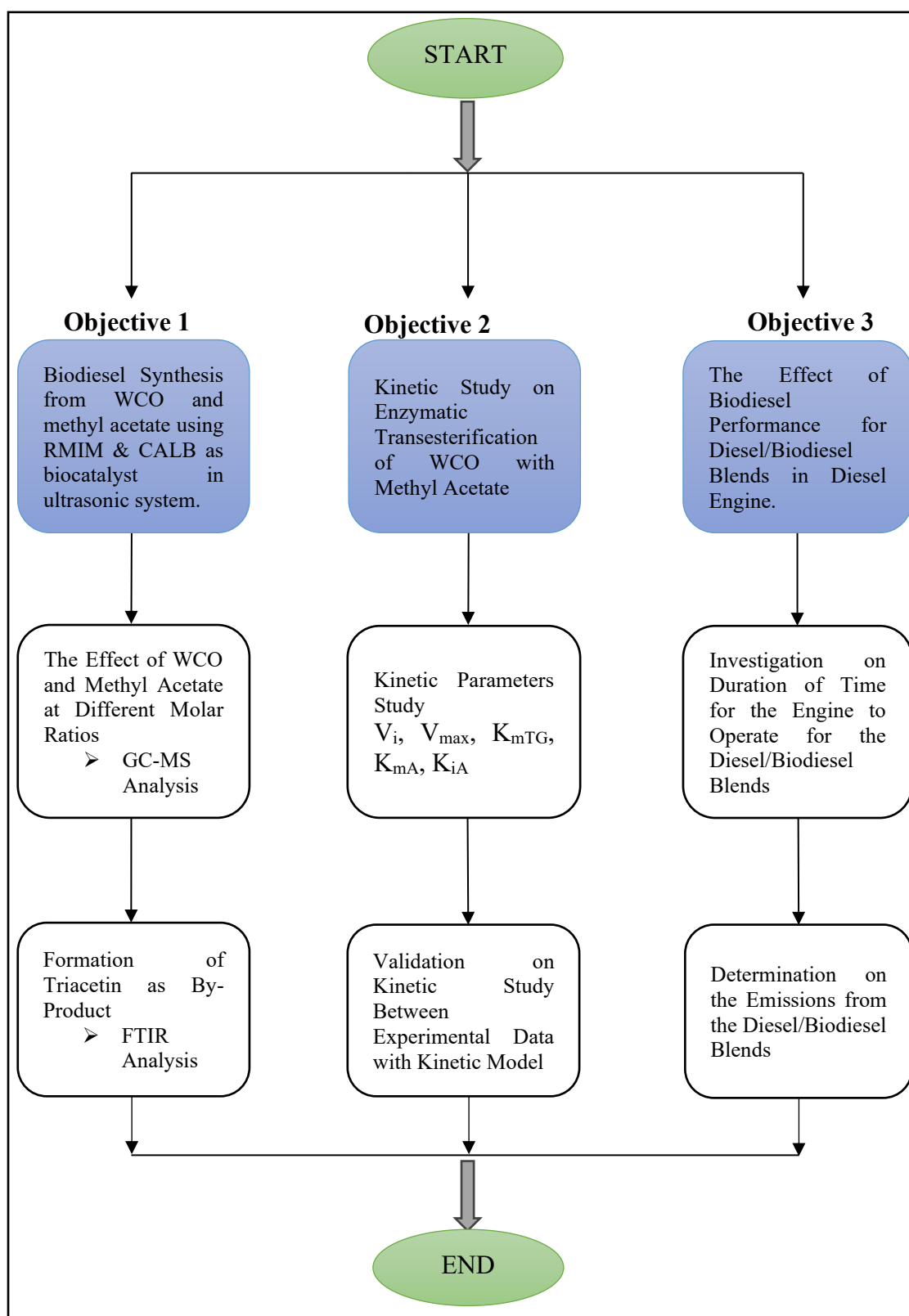


Figure 3.1 Overall Summary of Methodology

3.2 Materials and Chemical

Waste Cooking Oil (WCO) was used as the triglyceride feedstock for biodiesel synthesis in this study. The WCO was collected from domestic usage and stored in a clean 1.5 L polyethylene bottle before being transferred to the laboratory. The oil was kept at ambient laboratory conditions prior to experimental use. This feedstock was selected due to its availability and potential to reduce environmental pollution and raw material costs associated with biodiesel production.

Methyl acetate was employed as the acyl acceptor in the transesterification reaction. The chemical was obtained from laboratory stock and used without further purification. Methyl acetate was selected as an alternative to conventional methanol in order to eliminate glycerol formation and to produce triacetin as a value-added by-product, which can function as an additive to improve fuel properties.

3.3 Selection of Immobilized Lipases

Two commercially available immobilized lipases were used as biocatalysts in this work. Both enzymes were purchased from Sigma-Aldrich Malaysia and were used as received, without performing any immobilization procedure. The first enzyme was *Rhizomucor miehei* lipase immobilized on Immobead 150 (RMIM), supplied in granular form. According to the manufacturer's specifications, the enzyme had a minimum activity of 300 U/g, with a lot-specific activity of 515 U/g. The immobilized support provides structural stability and facilitates separation of the enzyme from the reaction mixture.

The second enzyme was *Candida antarctica* lipase B (CALB) immobilized on Immobead 150, produced as a recombinant enzyme from *Aspergillus oryzae*. The manufacturer reported a minimum activity of 1800 U/g, while the lot-specific activity used in this study was 3367 U/g.

The selection of RMIM and CALB was based on their commercial availability in immobilized form and their reported applicability in biodiesel production. No enzyme stability, deactivation, or reusability studies were conducted in this research. Therefore, the comparison between RMIM and CALB was limited to their catalytic performance in terms of biodiesel yield under identical reaction conditions. The more

effective enzyme was subsequently selected for further experimental and kinetic studies.

3.4 Enzymatic Transesterification of Waste Cooking Oil

The enzymatic transesterification of waste cooking oil with methyl acetate was carried out in a three-neck round bottom flask with a condenser and a thermometer. The reaction setup was placed in an ultrasonic water bath to provide ultrasonic irradiation during the reaction as depicted in Figure 3.3(a). Ultrasound was applied to enhance mass transfer between the immiscible reactants and to improve enzyme-substrate interaction.

Figure 3.2 shows the graphical abstract of biodiesel production using WCO and methyl acetate with the help of enzymes and ultrasound.

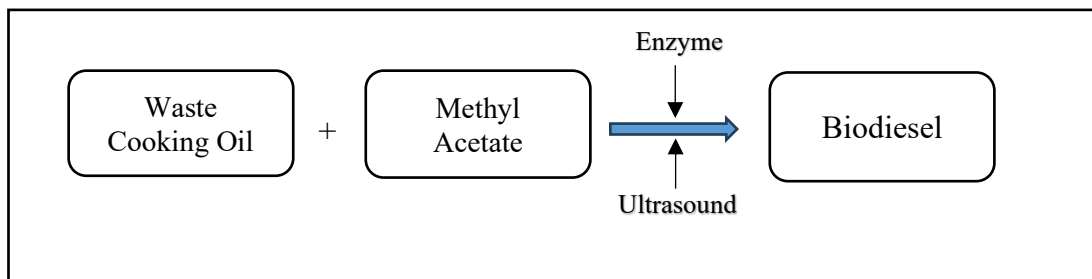


Figure 3.2 Graphical Abstract for Biodiesel Production

A standard reaction mixture was consisted of oil, methyl acetate and immobilized lipase. 10 grams of waste cooking oil and methyl acetate were mixed at stoichiometric molar ratio of waste cooking oil to methyl acetate of 1:3 at 45°C. The samples were analyzed for Free Fatty Acid (FFA) and run with GC-MS for biodiesel (FAME) analysis.

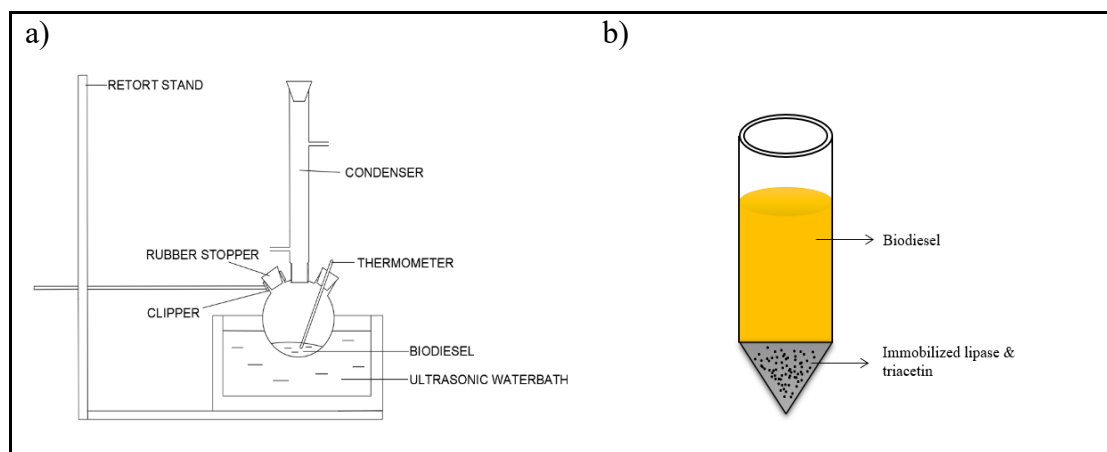


Figure 3.3 Experimental Setup (a) for Transesterification of Waste Cooking Oil and the Resulting Biodiesel Product (b)

A total of 18 w/w% of immobilized lipase was mixed with waste cooking oil and methyl acetate in a three-neck flask. The transesterification reaction was carried out for 3 hours. Upon completion, the resulting biodiesel mixture was centrifuged at 10,000rpm for 15 minutes to separate the enzyme and triacetin from the reaction mixture. After centrifugation, two distinct layers were observed, as illustrated in Figure 3.3(b), where the bottom layer contained the immobilized enzyme and triacetin, while the upper layer consisted primarily of the biodiesel product. The upper layer was carefully collected and subsequently analyzed using GC/MS to quantitatively determine the FAME yield.

3.5 Biodiesel Analysis

The biodiesel produced from the enzymatic transesterification of waste cooking oil with methyl acetate was analyzed to determine the fatty acid methyl ester (FAME) composition and to confirm the formation of triacetin as a by-product. Gas Chromatography–Mass Spectrometry (GC–MS) was employed to identify and quantify the methyl esters present in the biodiesel, while Fourier Transform Infrared (FTIR) spectroscopy was used to verify the presence of characteristic functional groups associated with triacetin.

3.5.1 Gas Chromatography-mass Spectrometry (GC/MS) Analysis

Gas Chromatography–Mass Spectrometry (GC–MS) analysis was carried out to determine the percentage and composition of methyl esters present in the biodiesel obtained from waste cooking oil. The GC–MS instrument used in this study is shown in Figure 3.4. Prior to analysis, the biodiesel samples were diluted with n-hexane to ensure appropriate concentration for injection into the GC system. All biodiesel analyses were performed in duplicate to ensure the reproducibility and reliability of the experimental results.



Figure 3.4 GC/MS Device

The GC–MS system was equipped with a Carbowax capillary column. Separation of the methyl ester components was achieved based on differences in volatility and polarity within the column. The resulting chromatographic peaks were compared with those obtained from a commercial FAME standard mixture (C₄–C₂₄) to identify the individual fatty acid methyl esters present in the biodiesel sample.

An external calibration approach was applied using the FAME standard mixture (C₄–C₂₄), in which the GC peak areas were correlated with known concentrations of the standard to support quantitative interpretation of the results. The calibration curve for FAME Mix, C₄–C₂₄ can be found in Appendices section (Appendix 1). The GC–MS analysis was primarily used to confirm FAME formation and to evaluate the relative distribution of methyl esters in the biodiesel product. The overall biodiesel yield was calculated gravimetrically based on the mass of biodiesel obtained relative to the initial mass of waste cooking oil, as expressed in Equation (3.1):

$$\text{Biodiesel (FAME) yield (\%)} = \frac{\text{Weight of Biodiesel (FAME) (g)}}{\text{Weight of Oil (g)}} \times 100\% \quad (3.1)$$

GC–MS is a powerful analytical technique widely used for the analysis of small organic molecules in various fields, including food, environmental, and petrochemical industries. In this technique, the sample is vaporized and transported through a capillary column by an inert carrier gas, where individual compounds are separated. Upon elution from the column, the compounds are ionized and fragmented in the mass spectrometer, and the resulting ions are detected based on their mass-to-charge (*m/z*) ratios. Compound identification is achieved by matching the mass spectra with reference libraries, while peak areas are proportional to the amount of each component present.

3.5.2 Fourier Transform Infrared (FTIR) Analysis

Fourier Transform Infrared (FTIR) spectroscopy was employed to confirm the formation of triacetin as a by-product of the interesterification reaction between waste cooking oil and methyl acetate. The FTIR instrument used in this study is shown in Figure 3.5 below.



Figure 3.5 FTIR Device

The analysis was conducted using Attenuated Total Reflectance (ATR) mode for liquid samples, and spectra were recorded in the range of $4000\text{--}400\text{ cm}^{-1}$. FTIR is a rapid and efficient method to identify functional groups based on their characteristic absorbance peaks. The presence of ester functional groups, indicative of triacetin, was confirmed through comparison of the observed peaks. FTIR analysis is a practical tool for supporting biodiesel characterization due to its simplicity and non-destructive nature (Rosset & Perez-Lopez, 2019).

3.6 Enzyme Kinetic Parameter Study

3.6.1 Enzyme Kinetic Study for Two Substrates

The enzyme kinetic study was conducted using CALB as biocatalyst in an ultrasonic system. Ten different concentrations of the substrates were tested, with the concentration of triglycerides (WCO) varied from 0.1054M to 1.0315M, and methyl acetate concentration varied from 0.089M to 11.3049M, while maintaining the total weight of substrates (WCO and methyl acetate) at a molar ratio of 1:12, at which the highest biodiesel yield was obtained using CALB in the preliminary optimization study. Other parameters of reaction were set at optimum conditions based on previous studies. The samples were collected at regular intervals from 0 to 30 minutes, and the biodiesel

yield was quantified using GC-MS. The reactions were carried out within the limit of less than 15% of transesterification.

3.6.2 Validation of Kinetic Parameter

The validation of the kinetic parameters was performed using Polymath software by comparing the experimental data with the Ping Pong Bi-Bi substrate competitive inhibition mechanism as proposed by Xu et al. (2005). This approach was employed to identify the best fit kinetic model describing the enzymatic transesterification of waste cooking oil (WCO) with methyl acetate. The general equation for Ping Pong Bi-Bi mechanism incorporating substrate competitive inhibition is expressed as follows:

$$V_i = \frac{V_{\max} [TG][A]}{K_{mTG}[A] \left(1 + \left(\frac{[A]}{K_i} \right) \right) + K_{mA}[TG] + [TG][A]} \quad (3.2)$$

Where V_i is the initial reaction rate, $V_{\max}$ is the maximum reaction rate, K_{mTG} is the Michaelis-Menten constant for triglyceride, K_{mA} is the Michaelis-Menten constant for alcohol and K_i is the inhibitor constant.

The selection of kinetic parameters was based on three criteria which are the highest correlation coefficient (R^2) between predicted and experimental data, a 95% confidence level for parameter estimates and random distribution of residuals around the error line.

3.7 Experimental Set-up for Diesel Engine Testing

In this study, the diesel engine performance tests were conducted using a Yanmar Diesel Bench Dynamometer. Biodiesel was blended Industrial Fuel (B7) and Commercial Fuel (B10) at varying proportions, reaching up to 20% by volume. The composition and details of each diesel/biodiesel blend are presented in Table 3.1.

Table 3.1
Diesel/biodiesel Blends and Its Percentage Blends

Diesel/Biodiesel Blends	Description
B20a	Blend of B7 with 13% of pure biodiesel from current study.
B20b	Blend of B10 with 10% of pure biodiesel from current study.

A 100 mL fuel sample was prepared and poured into the engine's fuel inlet. The torque was set to 1 Nm, and the engine was started. The engine speed was gradually increased to reach a medium level. Once the engine ran smoothly and reached a stable condition, the timer was started. Data from the gas analyzer, as well as the temperatures of the exhaust oil and intake air shown on the display panel, were recorded after 20 mL of the sample had been consumed. The same procedure was repeated when the fuel consumption reached 50 mL and 80 mL to obtain average values for analysis. Each fuel blend was repeated under identical engine operating conditions. Figure 3.6 shows the experiment set up (a) and diesel/biodiesel blends prepared (b).

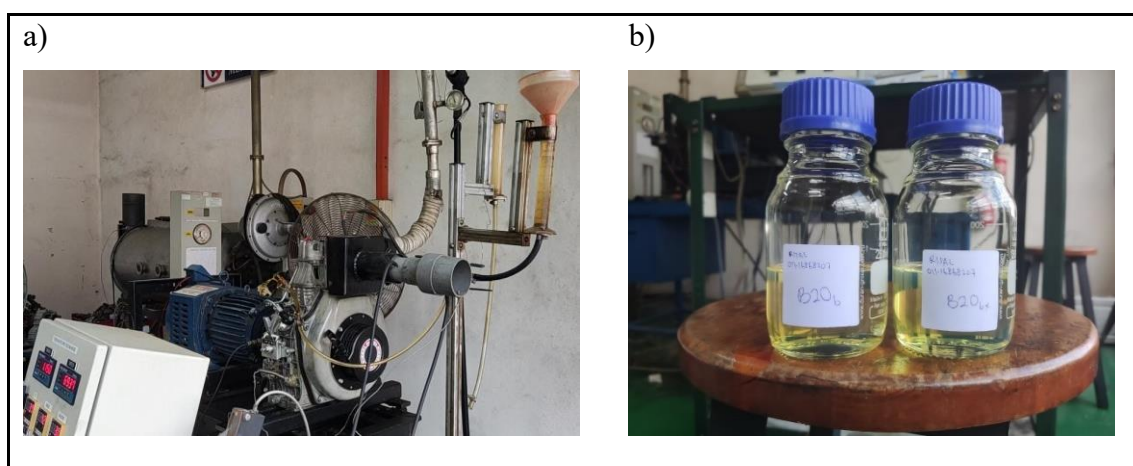


Figure 3.6 The Experiment Set-up (a) for the Diesel Engine and the Diesel/Biodiesel Blends (b)

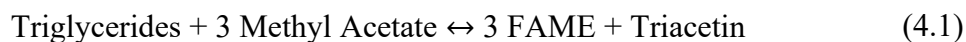
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Transesterification of WCO with Methyl Acetate Using Two Commercial Immobilized Lipase

Transesterification is a chemical reaction widely used in biodiesel production, involving the conversion of triglycerides into fatty acid methyl esters (FAME), typically sourced from vegetable oils or animal fats, in the presence of an alcohol or ester compound known as the acyl acceptor (Naseef & Tulaimat, 2025). In this study, waste cooking oil (WCO) was transesterified with methyl acetate using two commercial immobilized lipases. Enzymatic transesterification has been reported to offer several advantages over chemical catalysis, including operation under mild conditions, lower by-product formation, and easier product separation (Wong et al., 2023).

Stoichiometrically, one mole of triglyceride reacts with three moles of methyl acetate to form three moles of FAME and one mole of triacetin as illustrated in Equation (4.1):



In practical applications, an excess amount of methyl acetate is commonly employed to shift the reaction equilibrium toward biodiesel formation. As a non-alcohol acyl acceptor, methyl acetate participates in the transesterification reaction by converting triglycerides into FAME while simultaneously acetylating the glycerol backbone, leading to the formation of triacetin instead of free glycerol (Wong et al., 2023). The triacetin produced is fully miscible with biodiesel and does not accumulate on the enzyme surface, thereby reducing mass-transfer limitations and minimizing glycerol-related enzyme inhibition. Consequently, the use of methyl acetate was associated with sustained catalytic performance during the reaction, which was reflected in the higher FAME conversion observed in methyl acetate-based transesterification systems.

Based on this principle, experiments were conducted to identify the optimal molar ratio of Waste Cooking Oil (WCO) to methyl acetate for two types of commercial immobilized lipases, namely *Rhizomucor miehei* lipase (RMIM) and *Candida antarctica* lipase B (CALB) within a temperature controlled at 45°C, using 1.8wt% of

immobilized lipase. The biodiesel yield obtained from transesterification of waste cooking oil with methyl acetate at different molar ratios of WCO to methyl acetate (1:3 – 1:12) are shown in Figure 4.1.

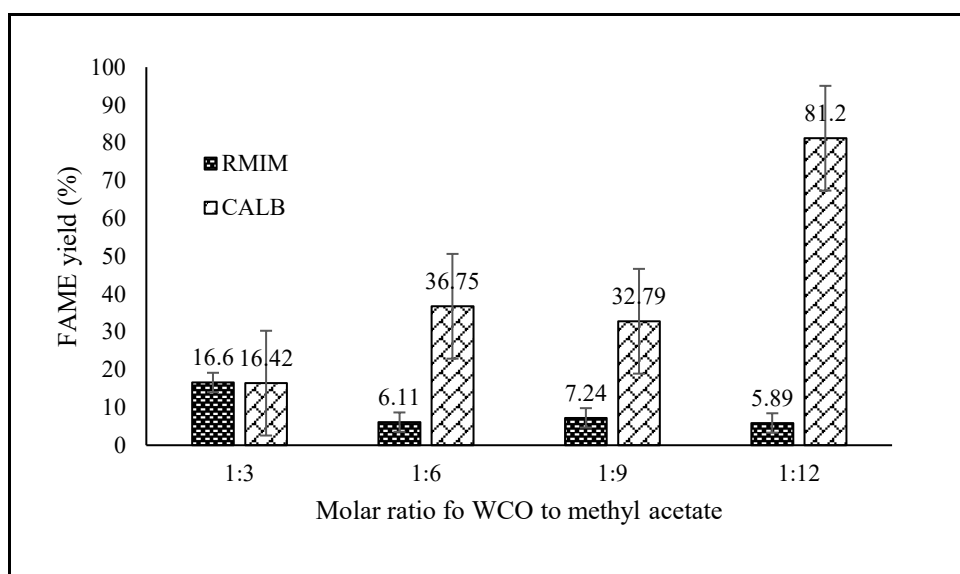


Figure 4.1 Biodiesel Yield Obtained from Transesterification of Waste Cooking Oil with Methyl Acetate Using Commercial Immobilized RMIM and CALB at Different Molar Ratios (1:3 – 1:12). Reaction Conditions: Temperature 45°C, Enzyme Loading 1.8% (w/v), Reaction Time 3h, Ultrasonic Assisted System at 5kHz

The experimental results revealed distinct trends in biodiesel yield for both immobilized lipases where the RMIM exhibited the highest biodiesel yield of 16.6% at molar ratio of 1:3 of WCO to methyl acetate. However, further increased in methyl acetate beyond this ratio led to a decline in FAME yield. This reduction was likely due to enzyme inhibition caused by an excessive amount of methyl acetate which reduced the contact of oil and methyl acetate (Zainal et al., 2023), suggesting that RMIM was sensitive to concentrations beyond the stoichiometric requirement.

In contrast, CALB showed a markedly different performance. A significant increase in biodiesel yield was observed with increasing methyl acetate, reaching a peak yield of 81.2% at the highest tested molar ratio of 1:12 of WCO to methyl acetate. This indicates that CALB remained catalytically active even in the presence of excess acyl acceptor and enhanced the conversion efficiency of triglycerides into biodiesel (Marín-Suárez et al., 2019). The surplus methyl acetate was contributed to better substrate availability and interaction with the enzyme, promoting a more complete transesterification process (Xu et al., 2005). A slight reduction in FAME yield was observed at a 1:9 molar ratio of WCO to methyl acetate (32.79%) compared to the 1:6

ratio (36.75%). Although an overall increase in biodiesel yield was generally expected with increasing molar ratio, this minor decrease could be attributed to a transient imbalance between substrate availability and enzyme saturation, which temporarily limited effective triglyceride conversion at this intermediate ratio. At the higher molar ratio of 1:12, sufficient substrate availability and enzyme activity were restored, resulting in the highest FAME yield.

The differences in biodiesel yields can also be partly explained by the intrinsic unit activities of the enzymes. CALB has a unit activity of 3367 U/g, whereas RMIM has a unit activity of 515 U/g. The higher activity of CALB indicated a greater number of active catalytic sites per unit mass of enzyme (Yu et al., 2021), which contributed to faster reaction rates and higher conversion efficiencies under identical reaction conditions (Gusniah et al., 2020). Conversely, the lower activity of RMIM limits its overall catalytic performance, which may partly explain the lower biodiesel yields observed.

The superior performance of CALB can also be attributed to its non-specific catalytic activity (Wong et al., 2023). CALB was capable of hydrolyzing ester bonds at all positions of the triglyceride molecule (Hwang et al., 2024), enabling the complete utilization of fatty acid chains during the reaction. As a result, a higher biodiesel yield was achieved, under a 1:12 molar ratio of WCO to methyl acetate at 45°C in an ultrasonic system, achieving a maximum FAME yield of 81.20%. On the other hand, RMIM exhibits positional specificity, primarily acting on the sn-1 and sn-3 positions of triglycerides (Shahedi et al., 2019). This limited action has left some fatty acid chains unreacted, thereby reducing the overall conversion efficiency and leading to lower biodiesel yields (Huang & Yan, 2008). Similar observations were reported by Marín-Suárez et al., (2019), who found that Novozym 435 (CALB) achieved a maximum yield of 82.91% significantly higher than Lipozyme RM IM and TL IM, which reached only 73-76% under comparable conditions. This study highlighted that the non-specific nature and robustness of CALB towards alcohol inhibition allowed for higher conversion efficiencies, while 1,3-specific lipases like RM IM were more sensitive to acyl acceptor concentrations, resulting in lower yields.

Although the yield with CALB showed a continuous increase up to the 1:12 molar ratio, further increasing the ratio was not pursued based on the facts that previous studies showed that an excess acyl acceptor (like methyl acetate) may not proportionally improve conversion and could even lead to enzyme inhibition or dilution of substrates

(K. S. Moreira et al., 2020). For instance, in a study by Amini et al., 2017, it was observed that increasing the molar ratio of fatty acids to alcohol initially enhanced biodiesel yield, but beyond a certain point such as 1:12 to 1:15, the conversion decreased. Similar trends were noted in studies with methanol, where Novozym 435 was deactivated at a molar ratio due to alcohol inhibition (Nayak & Vyas, 2019). Therefore, 1:12 was selected as the upper molar ratio in this study to balance yield, enzyme activity, and process feasibility. The yield comparison of biodiesel synthesized with CALB enzyme from current study with previous reported research was shown in Table 4.1.

Table 4.1
Yield Comparison of Biodiesel Synthesized with CALB Enzyme

No	Feedstock	Enzyme Loading (wt%)	Acyl acceptor	Molar ratio	Sonication	Biodiesel Yield (%)	Reference
1	Waste cooking oil	1.8	Methyl acetate	1:12 of oil to methyl acetate	Yes (5kHz)	81.20	Current study
2	Waste Cooking Oil	40	Methyl acetate	1:6 of oil to methanol	No	72.0	(Cerón Ferrusca et al., 2023)
3	Yellow horn seed	15	Methanol	1:1.6 of oil to methanol	No	92.3	(Zhang et al., 2020)
4	Waste Tallow	6	Methanol	1:4 of oil to methanol	Yes (5kHz)	85.6	(Adewale et al., 2016)
5	Soybean oil	30	Methyl acetate	1:12 of oil to methyl acetate	No	92	(Du et al., 2004)

The comparisons of biodiesel yield from various studies highlighted the influence of feedstock, enzyme loading, acyl acceptors and molar ratios on reaction efficiency. For current study, 81.20% of yield was achieved using WCO and methyl acetate with an enzyme loading of 1.8% w/v. This yield demonstrated the effectiveness of CALB in converting low-quality feedstock such as WCO into biodiesel. However, it was slightly lower than yield reported for cleaner feedstock such as yellow horn seed with 92.2% (Zhang et al., 2020), soybean oil with 92% (Du et al., 2004), and waste tallow with 85.6% (Adewale et al., 2015a). The type of feedstock plays an important

role in determining the yield. WCO usually contains impurities and high free fatty acid content which can inhibit enzymatic activity and reduce overall efficiency (Liu et al., 2021). In contrast, feedstocks such as yellow horn seed, soybean oil and castor oil were relatively cleaner, allowing for more efficient enzymatic reaction. Interestingly, the biodiesel yield for waste tallow that observed from (Adewale et al., 2016), was comparable to the yield achieved in the current study using waste cooking oil, despite differences in the physicochemical properties of the feedstocks and reaction conditions. Waste tallow was generally richer in saturated fatty acids and contains fewer polar degradation products compared to waste cooking oil (Adewale et al., 2016), which contained higher levels of free fatty acids, oxidation products, and food residues that may inhibit enzymatic activity.

Enzyme loading is another factor influencing biodiesel production yield. The current study employed the least enzyme loading from other studies with 1.8 wt%. While higher enzyme loading enhances catalytic activity and often results in higher yields (Vilas Bôas & Mendes, 2022), it also increases production costs. The biodiesel yield achieved in this study with minimal enzyme loading highlights a cost-effective approach that balances efficiency and economic feasibility.

The choice of acyl acceptor also impacts biodiesel production. Methyl acetate offers advantages over methanol due to its ability to prevent enzyme deactivation and maintain stability during the reaction (Norjannah et al., 2016). Although methanol was reported to produce higher yields with certain feedstocks, its polarity could lead to enzyme inhibition, denaturation and deactivation, particularly at high concentration. Studies employing methanol with stepwise addition achieved 92.3% of biodiesel yield as reported by Zhang et al., (2020) for yellow horn seed as feedstock.

Molar ratios of oil to acyl acceptor further influenced the reaction. A molar ratio of 1:12 was selected in the current study, ensuring sufficient acyl acceptor for complete conversion. However, lower molar ratios, as observed in the study by Zhang et al. (2020) with a 1:1.6 ratio, could optimize enzyme activity by reducing substrate inhibition. Our finding was comparable to Du et al., (2004), who used methyl acetate as acyl acceptor and molar ratio of oil to methyl acetate is 1:12.

Sonication plays a significant role in enhancing enzymatic biodiesel production by improving mass transfer and reaction kinetics. Ultrasonic irradiation generates acoustic cavitation, producing microbubbles that collapse violently and create localized high temperature and pressure zones (Gusniah et al., 2019). As shown in Table 4.1,

studies employing sonication generally achieved higher biodiesel yields at lower enzyme loadings compared to non-sonicated systems. In the current study, the application of sonication at 5 kHz contributed to achieving an 81.20% biodiesel yield from waste cooking oil using only 1.8 wt% of CALB, outperforming the non-sonicated system reported by Cerón Ferrusca et al., (2023), which required substantially higher enzyme loading yet resulted in a lower yield. A similar enhancement effect of sonication was observed in the work by Adewale et al., (2015a), where ultrasonic assistance enabled a high yield from waste tallow despite differences in feedstock composition.

Thus, based on the biodiesel yield obtained, CALB was identified as a suitable commercial immobilized lipase for biodiesel production from waste cooking oil using methyl acetate under ultrasonic conditions.

4.2 Formation of Triacetin as By-product

During enzymatic transesterification using methyl acetate as acyl acceptor, not only biodiesel is produced but also triacetin as a valuable by-product. According to Dhawan et al., (2021) and Pastres et al., (2022), triacetin is a valuable by-product formed during the transesterification of triglycerides with methyl acetate. It is considered more commercially valuable than glycerol due to its higher carbon content and energy density, which make it useful as a fuel additive, as well as its versatility in applications such as pharmaceuticals and cosmetics (Zada et al., 2022; Zare et al., 2016). The concentration of triacetin can vary depending on the molar ratio of WCO to methyl acetate applied during the reaction. At lower molar ratios, the availability of methyl acetate may limit the formation of triacetin while higher molar ratios provide sufficient reactant to promote secondary reactions leading to greater triacetin production.

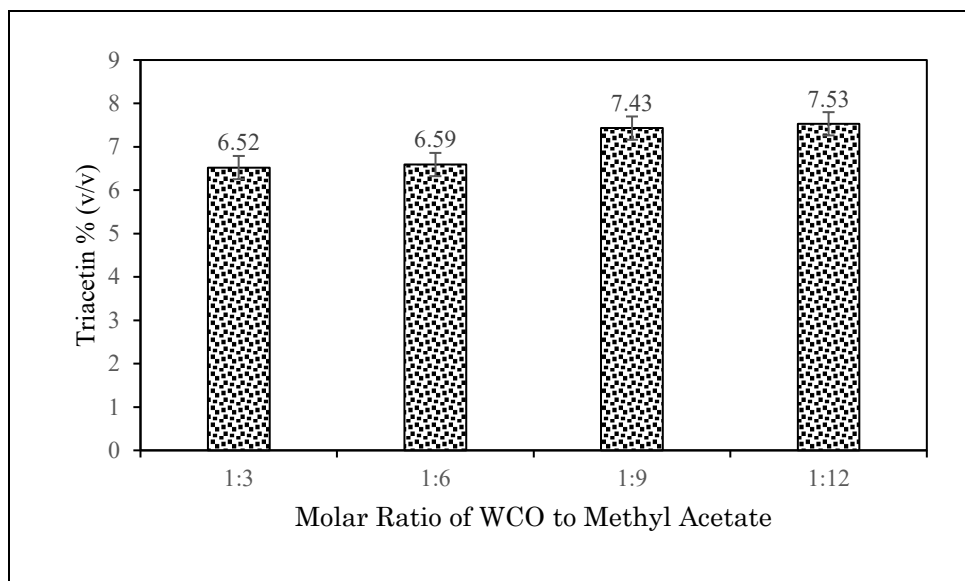


Figure 4.2 Effect of Molar Ratio on Triacetin Concentration

In this study, the concentration of triacetin as by-product was determined for each molar using CALB, selected based on its higher biodiesel yield compared to RMIM. As shown in Figure 4.2, the concentration of triacetin increases progressively with higher molar ratios of WCO to methyl acetate. This trend was attributed to the role of excess methyl acetate which drives the reaction toward the completion and enhances the formation of both main product (biodiesel) and by-product (triacetin). As the availability of methyl acetate increases, it promotes the subsequent reactions that lead to higher triacetin production (Tan et al., 2011).

The formation of triacetin was further confirmed through FTIR analysis. Figure 4.3 presents the comparison of IR spectra for enzymatic transesterification of WCO with methyl acetate and WCO, allowing direct comparison of absorption bands and clear identification of structural differences associated with triacetin formation.

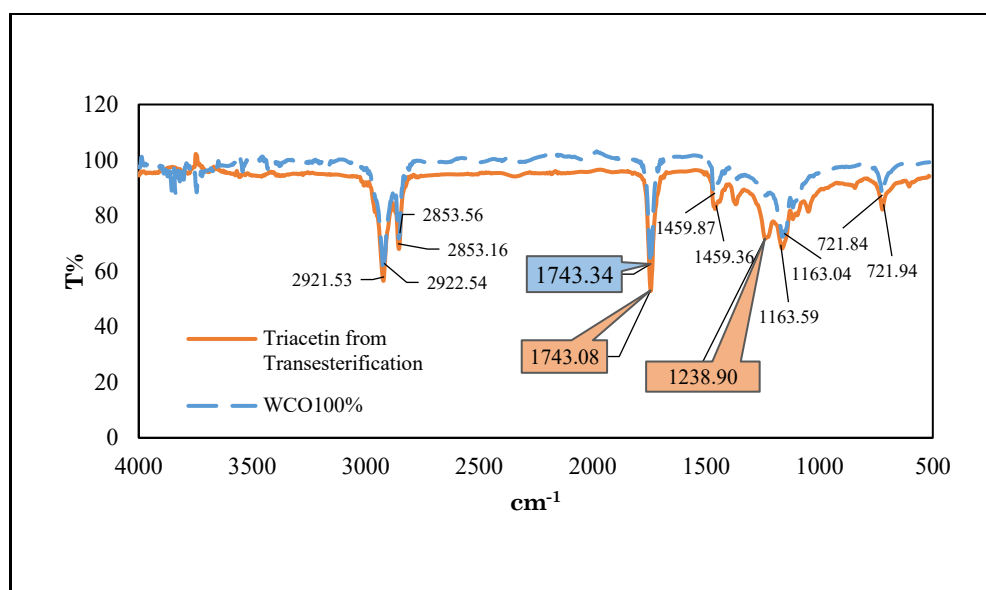


Figure 4.3 Comparison of IR Spectra for Enzymatic Transesterification of WCO with Methyl Acetate and pure WCO

The FTIR absorption bands, functional group assignments, vibration types, and absorption intensities for both enzymatic transesterification of WCO and methyl acetate with pure WCO were summarized in Table 4.2.

Table 4.2

IR Interpretation of Triacetin from Enzymatic Transesterification of WCO with Methyl Acetate

Sample	Group Attribution	Wavenumber (cm ⁻¹)	Absorption Intensity
Triacetin	-CH ₂	2921.53	Strong
	-CH ₂	2853.16	Strong
	-C=O	1743.08	Strong
	-CH ₂	1459.36	Middling
	-C=O	1238.90	Middling
	C-O-C	1163.59	Middling
	-CH ₂	721.94	Weak
WCO	-CH ₂	2922.54	Strong
	-CH ₂	2853.56	Strong
	-C=O	1743.34	Strong
	-CH ₂	1459.87	Middling
	C-O-C	1163.04	Middling
	-CH ₂	721.84	Weak

From Table 4.2, both spectra exhibited strong absorption bands at approximately 2921–2923 and 2853 cm⁻¹, which corresponded to asymmetric and symmetric stretching vibrations of aliphatic –CH₂ groups, respectively. These bands were

characteristic of long-chain fatty compounds and were therefore present in both WCO and the reaction product. A strong absorption band was observed at around 1743 cm^{-1} in both spectra and was attributed to ester carbonyl ($\text{C}=\text{O}$) stretching vibrations. The presence of this band in pure WCO originated from triglyceride ester linkages, while its persistence in the product spectrum indicated the retention of ester functional groups following enzymatic transesterification. However, the ester carbonyl band in the region of $1700\text{--}1750\text{ cm}^{-1}$ could not be considered a diagnostic indicator for triacetin formation, as it overlapped with the $\text{C}=\text{O}$ stretching vibrations of fatty acid methyl esters and triglycerides already present in WCO. Similar observations were reported by Pastres et al., (2022), who demonstrated that although triacetin contributed a peak near 1750 cm^{-1} , this absorption overlapped with existing ester signals and therefore could not uniquely confirm the presence of triacetin.

Despite the similarity of the major absorption bands, the overlaid spectra in Figure 4.3 revealed a clear distinction in the fingerprint region. A distinct absorption band at 1238.90 cm^{-1} was observed exclusively in the spectrum of triacetin and was absent in the spectrum of pure WCO. This band was assigned to $\text{C}\text{--}\text{O}$ stretching vibrations of ester groups, specifically associated with acetate ester linkages formed during the conversion of triglycerides into triacetin. According to Pastres et al., (2022), absorption bands in the range of $1200\text{--}1250\text{ cm}^{-1}$ were characteristic and unique to triacetin and were absent in compliant diesel, biodiesel containing fatty acid methyl esters, and other potential ester-containing additives. Therefore, this region serves as a selective and reliable indicator for triacetin detection.

While most absorption bands were common to both samples due to their ester-based molecular structures, the presence of the $\text{C}\text{--}\text{O}$ stretching band at approximately 1239 cm^{-1} exclusively in the triacetin spectrum served as the key diagnostic feature confirming triacetin formation. Consistent with previous studies (Pastres et al., 2022; Zainal et al., 2023), the emergence of this characteristic band, together with the observed increase in triacetin concentration at higher methyl acetate molar ratios, confirmed the successful formation of triacetin via enzymatic transesterification in the present study.

4.3 Enzyme Kinetic Study

The enzyme kinetics for the transesterification of WCO with methyl acetate using immobilized *Candida antarctica* lipase B (CALB) were investigated. From the initial reaction rate, it was identified as the slope of the initial product formed over time.

The enzymatic reaction rate was determined by varying ten different concentrations of the two substrates: triglyceride (TG) and methyl acetate (A) while maintaining a constant total weight of 20.146g, corresponding to the optimal molar ratio of 1:12 of CALB and labelled as sample [A] to [J]. For instance, sample [A] consisted of 2 grams of WCO and 18.148 grams of methyl acetate while sample [J] consisted of 20 grams WCO and 0.146grams of methyl acetate. The concentrations of triglyceride were varied from 0.1054M to 1.0315M while concentrations of methyl acetate were varied from 0.089M to 11.3049M. The details of varied concentration for both triglycerides and methyl acetate were tabulated in Table 4.3.

Table 4.3
Varied Concentrations of Triglyceride and Methyl Acetate

Sample	Concentration of Triglyceride, [TG] (M)	Concentration of Methyl Acetate, [A] (M)
[A]	0.1054	11.3049
[B]	0.2102	10.0349
[C]	0.3146	8.7709
[D]	0.4185	7.5129
[E]	0.5218	6.2610
[F]	0.6247	5.0149
[G]	0.7271	3.7747
[H]	0.8291	2.5404
[I]	0.9305	1.3118
[J]	1.0315	0.0890

From these ten samples, five representative concentrations covering the relevant substrate range were selected for kinetic data regression. The selected samples were then used in Polymath 6.10 software to perform non-linear regression analysis based on the Ping Pong Bi-Bi mechanism, incorporating competitive inhibition by methyl acetate. The Levenberg-Marquardt (LM) algorithm, applied by default in Polymath, was used to optimize the kinetic constants. Initial guesses for V_{max} , K_{mTG} , K_{mA} and K_{iA} , were required to initiate the regression. The optimized values obtained from curve fitting were $V_{max}=0.000026 \text{ Mmin}^{-1}$; $K_{mTG}= 0.00496\text{M}$; $K_{mA}=0.057\text{M}$; $K_i=0.178\text{M}$. The model fit exhibited a high coefficient of determination, with $R^2= 0.99$ and $R^2_{adj}=0.97$, confirming the accuracy of the model.

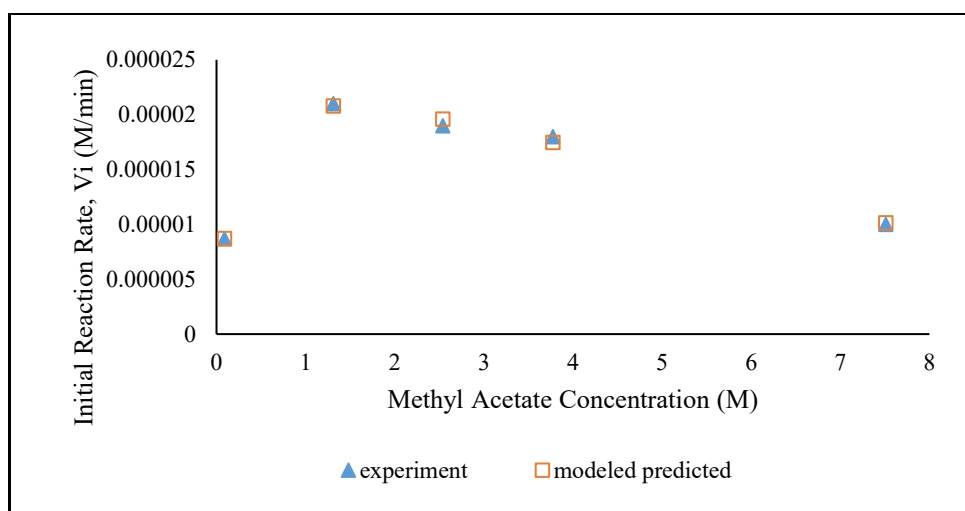


Figure 4.4 Comparison of Model Prediction and Experimental Data in Ultrasonic System. Reaction Conditions: 20.146 g of Mixture of WCO and Methyl Acetate, 1.8 wt% of CALB, 45°C

Figure 4.4 illustrated the comparison between the predicted values from the kinetic model and the corresponding experimental data. The model predictions were in a good agreement with the experimental data, indicating strong consistency between the simulated and observed results. As shown in the Figure 4.4, the initial reaction rate rose with increasing methyl acetate concentration up to approximately 1.31 M. Beyond this concentration, a gradual decline in the initial reaction rate was observed. This decline was attributed to substrate inhibition, where excessive methyl acetate began to negatively affect the reaction rate.

The enzymatic transesterification in this study followed a Ping-Pong Bi-Bi mechanism with competitive inhibition, in which one substrate (WCO) bound to the enzyme to form a fatty acyl-enzyme intermediate, releasing a partial glyceride product. The modified enzyme then reacted with the second substrate (methyl acetate) to produce

fatty acid methyl esters, regenerating the free enzyme, as described by Ruzich & Bassi, (2011). Competitive inhibition by methyl acetate was incorporated into the kinetic model to account for substrate interactions and the observed decrease in reaction rate at high acyl acceptor concentrations. The presence of the inhibition constant (K_i) further supported this observation, as it quantified the extent to which methyl acetate impeded the enzymatic activity (Xu et al., 2005). The kinetic constants obtained in this study differed from those reported in similar studies applying the same or comparable models. Such variations were expected due to the high sensitivity of kinetic parameter determination to experimental reaction rates (Ruzich & Bassi, 2011). Even slight deviations in experimental conditions could result in significantly different kinetic values, highlighting the complexity of modelling enzymatic reactions (Veny et al., 2014).

Therefore, the proposed enzyme kinetic for enzymatic transesterification of waste cooking oil and methyl acetate using CALB as biocatalyst in ultrasonic system can be stated as:

$$v_i = \frac{0.000026[TG][A]}{0.00496[A] \left(1 + \left(\frac{[A]}{0.178} \right) \right) + 0.057[TG] + [TG][A]} \quad (4.2)$$

Table 4.4

Comparison of Kinetic Parameters From Selected Studies on Lipase-catalyzed Transesterification via the Ping Pong Bi-Bi Mechanism with Substrate Competitive Inhibition

Studies	Lipases	Reactors	T (°C)	V _{max} (Ms ⁻¹)	K _{mTG} (M)	K _{iA} (M)	K _{mA} (M)
Current studies	Novozym 435 (CALB)	Ultrasonic system	45	0.00000043	0.0050	0.057	0.178
Veny et al., (2014).	Lipozyme RM IM	CBPBR	45	0.0000037	0.0067	0.042	2.212
Dossat et al., (2002).	Lipozyme RM IM	Glass tube stirrer	40	0.0000042	0.0053	0.055	0.013
Xu et al., (2005)	Novozym 435	Batch shaker	40	0.03167	1	0.0455	16
Halim & Harun Kamaruddin, (2008)	Novozym 435	Batch shaker	40	0.0156	2.61	10.25	1.6

The enzyme kinetics of transesterification in biodiesel production vary widely based on enzyme type, reaction conditions, and substrates, as seen in this study and comparative literatures. In this study, the maximum reaction rate values of V_{max} were lower than the values from all studies reported Xu et al., (2005); Halim & Kamaruddin (2008), Veny et al. (2014), and Dossat et al., (2002). According to Veny et al. (2014), the difference in the values was possibly due to different lipases used. Other than that, the ultrasonic system may have affected the enzyme stability and activity of the enzyme due to shear or thermal stresses (Qian et al., 2023).

In this study, K_{mTG} value indicated a higher substrate affinity for triglycerides compared to methyl acetate, as reflected in the K_{mA} value. This result aligned with the Ping Pong mechanism, which triglycerides were the first substrate to bind to the enzyme rather than methyl acetate (Ruzich & Bassi, 2011). This prioritization was due to the enzyme's greater affinity for triglycerides as confirmed by the lower K_{mTG} value relative to K_{mA} . Consequently, the reaction began with the binding of triglycerides to the lipase, forming an enzyme-substrate complex, before subsequent binding and conversion of the methyl acetate substrate. Similar trends were reported by Xu et al. (2005), who observed that lower K_{mTG} compared to K_{mA} reflected the enzyme's preferential binding to triglycerides in the transesterification reaction using Novozym 435 under the Ping Pong mechanism. In comparison to previous studies, the K_{mTG} value in this work was comparable to Dossat et al. (2002), and slightly better than Veny et al. (2014). In contrast, much higher K_{mTG} values were reported by Xu et al. (2005), and Halim & Kamaruddin (2008), suggesting lower triglyceride affinity in their systems. Regarding K_{mA} , this study reported moderate affinity for methyl acetate. This value was significantly lower than Veny et al. (2014), which implied better methyl acetate interaction in this system. However, it is higher than the K_{mA} value observed by Dossat et al. (2002), indicating less affinity compared to their system. Xu et al. (2005) and Halim & Kamaruddin (2008), reported much higher K_{mA} , suggesting reduced substrate interaction efficiency.

The inhibition constant K_{iA} in this study indicates moderate resistance to competitive inhibition. It was slightly higher than those reported by Dossat et al. (2002) and Veny et al. (2014), except from Halim & Kamaruddin (2008). They reported a much larger K_{iA} , which reflects significantly higher tolerance to inhibition in their system. The results were comparable to the findings from Dossat et al. (2002), who used butanol as acyl acceptor, as shown in Table 4.4.

4.4 Biodiesel Performance in Diesel Engine

4.4.1 Duration of Running Time for the Diesel Engine

The evaluation of diesel engine operation duration for different diesel/biodiesel blends provides valuable insights into fuel performance and efficiency. Biodiesel, derived from renewable sources, has gained attention as a sustainable alternative to fossil-based diesel due to its lower carbon footprint and biodegradable nature. However, its physical and chemical properties differ from conventional diesel, which can influence engine performance parameters such as combustion efficiency, fuel consumption, and operating duration (Gautam & Kumar, 2020). One of the key factors affecting engine operation time is the energy content of the fuel. Biodiesel generally has a lower energy content per unit volume compared to petroleum diesel, which may result in shorter operation durations when used in higher proportions (Rajendran et al., 2023). Additionally, differences in air–fuel mixing and volumetric efficiency when using biodiesel blends can influence combustion behaviour and fuel usage (Rahim et al., 2021), thereby affecting engine running time.

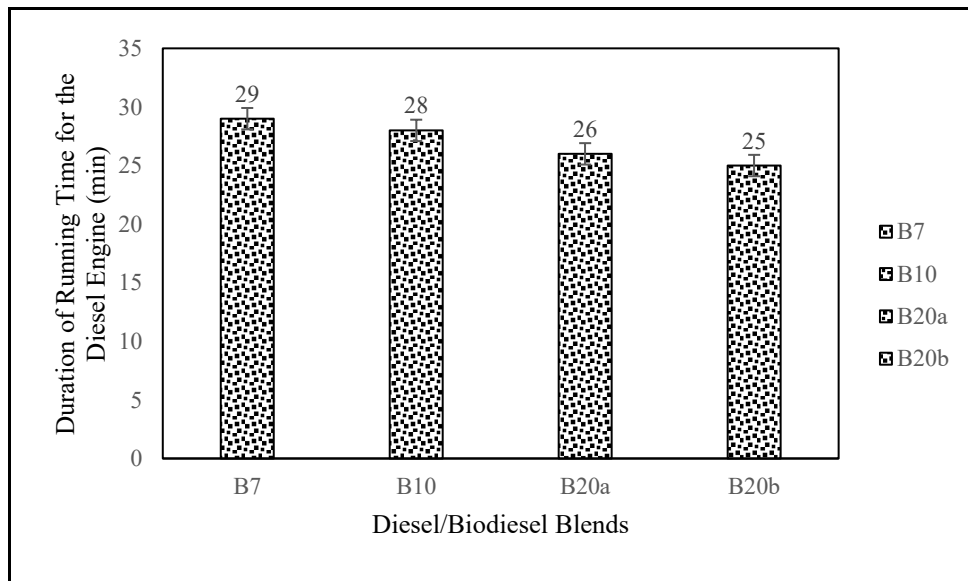


Figure 4.5 Duration of Running Time for the Diesel Engine

In this study, the engine operation duration was evaluated for different fuel blends, including B7 (industrial fuel), B10 (commercial fuel), B20a, and B20b. From Figure 4.5, the engine operation duration for B7 (industrial fuel) was recorded at 29 minutes, while B10 (commercial fuel) was rec at 28 minutes. This minor decrease was primarily attributed to the lower energy content of B10 compared to B7, which required

a higher fuel consumption rate to maintain similar engine performance. When biodiesel was introduced into the blends, the operation times decreased further, the B20a blend resulted in a duration of 26 minutes, while B20b recorded 25 minutes.

These shorter operating times were consistent with the fundamental properties of biodiesel, which has a lower calorific value than petroleum diesel (Siddeg et al., 2022). Consequently, for the same volume of fuel, less energy was available, leading to increased brake specific fuel consumption (BSFC) and faster fuel depletion. The higher viscosity of biodiesel also contributed to minor reductions in combustion efficiency, further influencing fuel consumption and operating duration. Overall, the observed trend confirmed that the addition of biodiesel in diesel blends reduced the engine running time, aligning with previous literature reports on biodiesel–diesel blends from (Rahim et al., 2021; Siddeg et al., 2022).

4.4.2 Effect of Biodiesel on NO_x Emission

Nitrogen oxides (NO_x) are among the primary pollutants emitted during diesel engine combustion, significantly impacting air quality and human health. The formation of NO_x in diesel engines is influenced by several factors such as high flame temperature, peak cylinder pressures, the nitrogen content of the fuel and the residence time of the fuel-air mixture within the cylinder (Xue et al., 2011).

In this study, exhaust emissions including carbon monoxide, nitrogen oxides and carbon dioxide were measured using a gas analyzer (MRU Vario Plus). A common observation in previous literature is the tendency for NO_x emissions to rise when biodiesel is introduced into diesel fuel blends. As shown in Figure 4.6, blending 10% of pure biodiesel with commercial fuel (B10) caused a slight increase in NO_x emissions, from 135ppm to 137ppm. This increase was likely due to the higher oxygen content present in biodiesel, which enhanced combustion and raised the temperature within the combustion chamber, thereby facilitating NO_x formation (Adaileh & Alqdah, 2012).

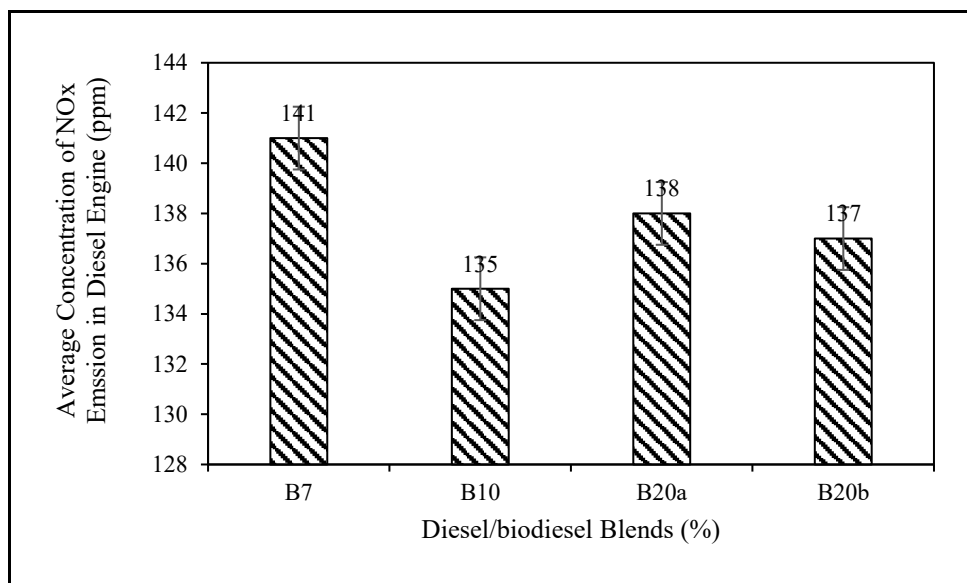


Figure 4.6 Average Concentration of NOx Emissions in Biodiesel/Diesel Blends. (B7: Industrial Fuel, B10: Commercial Diesel Fuel, B20a: B7 + 13% Biodiesel, B20b: B10 + 10% of Biodiesel)

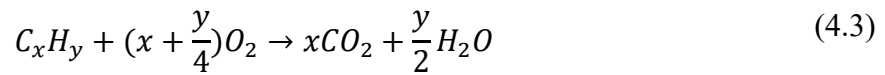
In contrast, the average concentration of NOx emissions was lower when 13% of pure biodiesel was blended with industrial fuel (B7) from 141ppm to 138ppm. These variations were attributed to the characteristics of biodiesel. Biodiesel typically has a higher cetane number and lower flash point than conventional diesel, as noted by Khujamberdiev & Cho, (2021). A higher cetane number reduces ignition delay, thereby decreasing the size of the premixed combustion phase. This leads to a slower rise in combustion pressure, allowing more time for cooling through heat transfer and dilution, which in turn lowers localized gas temperatures and reduces NOx formation rates (Khujamberdiev & Cho, 2021).

Furthermore, biodiesel lacks of aromatic and polyaromatic hydrocarbons, which contribute to lower flame temperatures. This characteristic further reduces the formation of NOx (Khujamberdiev & Cho, 2021). In addition, the shorter ignition delay caused by the higher cetane number allows less time for air-fuel mixing before the premixed burning phase, resulting in a weaker and less intense mixture. Consequently, the combustion process generates lower NOx emissions (Kalligeros et al., 2003; Knothe et al., 2006). Other than that, nitrogen oxides (NOx) emissions are also influenced by fuel type and blending concentration. According to Stanescu et al., (2025), NOx emissions vary with both the composition and proportion of biodiesel in the blend. In their study on sunflower oil biodiesel blends, NOx emissions generally decreased with increasing biodiesel content with the SF20 blend exhibited lower NOx emissions than

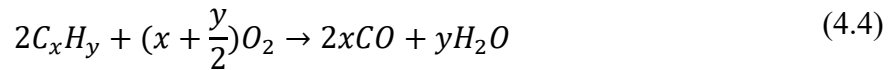
SF10. Similar cases happened which reported by (Arumugam A & Ponnusami V, (2017) and Adaileh & Alqdah, (2012).

4.4.3 Effect of Biodiesel on CO Emission

Carbon monoxide (CO) is colorless, odorless and considered as an intermediate product of hydrocarbon combustion (Ogunkunle & Ahmed, 2021). CO is a gas result from insufficient amount of oxygen reacting with all the fuel's carbon to produce CO₂, the unburned fuel is the cause of production of carbon monoxide (Jalaludin et al., 2020). The general reaction for the complete combustion of a hydrocarbon fuel can be represented in Equation (4.3). The equation represented the complete oxidation of carbon, where all carbon atoms are converted to CO₂, resulting in minimal CO formation.



Under oxygen-limited conditions, incomplete combustion occurred, producing CO instead of CO₂ as shown in Equation (4.4). The equation shows that when fuel and oxygen are not fully mixed, carbon is only partially oxidized, leading to CO formation (Kamil et al., 2019).



In diesel engines, CO emissions are influenced by several factors such as the air-fuel ratio, combustion temperature and oxygen availability (Xue et al., 2011). Biodiesel, due to its higher oxygen content, generally promotes more complete combustion compared to conventional diesel, potentially resulting in lower CO emissions (Arumugam A & Ponnusami V, 2017). Other than that, type of feedstock utilized in biodiesel production can also affect CO emissions. Wu et al., (2009), reported that biodiesel derived from different feedstocks exhibited varying oxygen contents and cetane numbers, which affected combustion efficiency and CO emissions. Based on Figure 4.7, the results showed significant differences between the two scenarios.

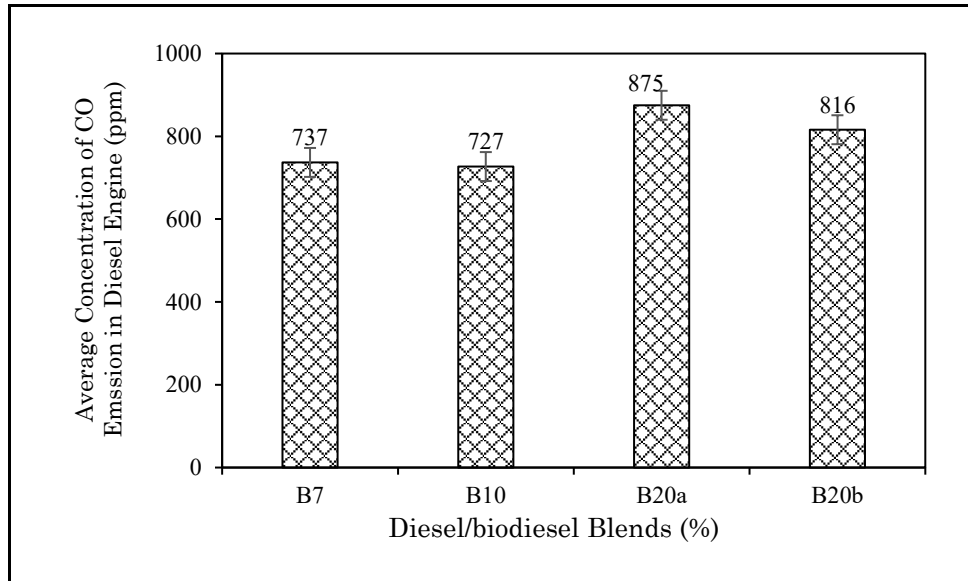


Figure 4.7 Average Concentration of CO Emissions in Biodiesel/Diesel Blends. (B7: Industrial Fuel, B10: Commercial Diesel Fuel, B20a: B7 + 13% Biodiesel, B20b: B10 + 10% of Biodiesel)

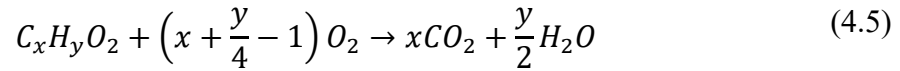
The average concentration of CO increased when 13% of pure biodiesel was blended with industrial fuel (B7) from 737ppm to 875ppm. Similarly, when 10% of pure biodiesel is blended with Commercial fuel (B10), CO emissions increased from 727 ppm to 816 ppm. While the CO emissions for B20b were lower than those for B20a, but they were still elevated. This considerable rise in CO concentration in the exhaust gases was attributed to the higher viscosity and poor spray characteristics of biodiesel which resulted in poor mixing and poor combustion (Arumugam & Ponnusami, 2017; Yunus et al., 2013). According to the incomplete combustion equation, these conditions promoted partial oxidation of carbon, resulting in higher CO formation instead of CO₂ (Kamil et al., 2019).

Additionally, the differences in base fuels (B7 and B10) may have contributed to the observed differences. B7 may have a higher aromatic content, which is known to affect combustion negatively, potentially leading to higher CO emissions. For B10, with its slightly higher cetane number, may promote better ignition and combustion, resulting in relatively lower CO emissions in B20b compared to B20a.

4.4.4 Effect of Biodiesel on CO₂ Emission

Carbon dioxide (CO₂) is a significant greenhouse gas produced during the combustion of hydrocarbon fuels (Kamil et al., 2019). As the ideal output of any hydrocarbon combustion process, CO₂, along with water, served as an indicator of

combustion efficiency. The combustion of an oxygenated fuel such as biodiesel can be described by the reaction in Equation (4.5).



Equation (4.5) represents the complete oxidation of carbon to CO₂ and hydrogen to water, indicating efficient combustion (Kamil et al., 2019). Although biodiesel emits carbon dioxide comparable to ordinary fossil fuel during combustion, the use of plant feedstock within the production process usually absorbs a certain quantity of atmospheric carbon dioxide while growing (Aljaafari et al., 2022). Studies estimate that biodiesel produced from waste cooking oil or fat reduces carbon dioxide emission by approximately 85% (Serrà et al., 2020). In addition, biodiesel combustion usually emits negligible sulphur dioxide emission and reduced levels of polyaromatic hydrocarbons, carbon monoxide and soot, further underscoring its environmental advantages (García-Martín et al., 2019).

Higher CO₂ emissions are typically associated with more complete combustion, as the carbon content in the fuel is fully oxidized (Mengistu et al., 2024). However, CO₂ emissions are often considered less harmful to human health, as they do not directly cause adverse health effects, although they are a major contributor to greenhouse gas emissions (Stanescu et al., 2025). The CO₂ emissions are primarily influenced by factors such as the compression ratio and exhaust gas temperature (Yaqoob et al., 2021). Biodiesel, with its higher oxygen content compared to conventional diesel, generally facilitates more complete combustion. This leads to greater oxidation of unburned hydrocarbons and carbon atoms, resulting in increased CO₂ emissions (Khujamberdiev & Cho, 2021). Many studies have reported that blending biodiesel with conventional diesel tends to raise CO₂ emissions due to its oxygen-rich molecular structure, which enhances combustion efficiency (Yaqoob et al., 2021). These studies indicate that higher biodiesel content in blends correlates with an increase in CO₂ emissions as a result of improved combustion characteristics. However, this study findings proved otherwise.

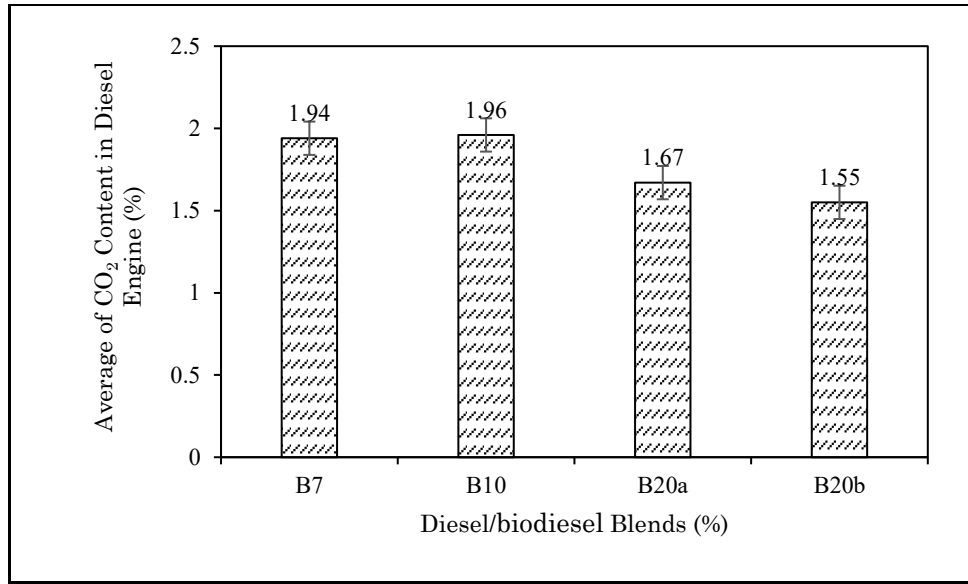


Figure 4.8 Average of CO₂ Content in Biodiesel/Diesel Blends (B7: Industrial Fuel, B10: Commercial Diesel Fuel, B20a: B7 + 13% Biodiesel, B20b: B10 + 10% of Biodiesel)

From Figure 4.8, the average of CO₂ content decreased when 13% of pure biodiesel was blended with industrial fuel (B7) from 1.94% to 1.64%. Similarly, when 10% of pure biodiesel was blended with commercial fuel (B10), CO₂ content decreased from 1.96% to 1.55%. The lower CO₂ emissions observed were primarily attributed to the lower carbon-to-hydrogen ratio of biodiesel compared to conventional diesel fuel (Mathiyazhagan et al., 2022; Sekhar & Gupta, 2025). A lower carbon content per unit fuel resulted in reduced CO₂ formation during combustion, as described by the general combustion equation in Equation (4.2) (Xue et al., 2011).

In addition, the reduction in CO₂ emissions was further explained by the life cycle carbon balance of biodiesel. Biodiesel produced from waste cooking oil was derived from biomass that had absorbed atmospheric CO₂ during its growth phase, thereby partially offsetting the CO₂ released during combustion (Stanescu et al., 2025). Life cycle assessment studies have estimated that biodiesel derived from waste cooking oil or fats can reduce CO₂ emissions by approximately 85% compared to fossil diesel (Aljaafari et al., 2022). The observed decrease in CO₂ emissions therefore indicated a potential environmental benefit, as lower CO₂ output contributes to mitigating greenhouse gas emissions while maintaining acceptable combustion performance.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In conclusion, the objectives of this study were successfully accomplished. Firstly, the catalytic performance of two commercial immobilized lipases in the transesterification of waste cooking oil (WCO) with methyl acetate under ultrasonic conditions was evaluated. Through comprehensive experimentation, it was found that *Candida antarctica* lipase B (CALB) achieved the highest biodiesel yield compared to *Rhizomucor miehei* (RMIM) with 81.2% at a molar ratio of 1:12 and 16.6% at a molar ratio of 1:3. The higher yield of CALB was attributed to its non-specific activity, whereas RMIM's specificity towards the sn-1,3 positions of triglycerides limited its conversion efficiency.

Secondly, the kinetic parameters of CALB in ultrasonic transesterification were determined. The enzyme kinetic study showed that the lipase kinetic was followed Ping Pong Bi-Bi mechanism with reversible inhibition by methyl acetate. Key kinetic parameters were determined: $V_{max}=0.000026\text{Mmin}^{-1}$, $K_{mTG}=0.0050\text{M}$, $K_{mA}=0.178\text{M}$ and $K_{iA}=0.057\text{M}$. These findings were compared with the proposed model and there was a good agreement between the experimental results of the initial reaction rate of reaction and those predicted by the model equation.

The performance evaluation of biodiesel blends in diesel engine revealed that each blend exhibited different emission and runtime characteristics. Using 200mL of biodiesel blend, B7 had the longest engine running time with 29 minutes, followed by B10, B20a and B20b indicating that higher biodiesel blend content slightly reduced operating time. In terms of emissions, B20a showed the lowest NOx emission compared to other blends. CO emissions increased with biodiesel blend addition, from 737ppm (B7) to 875ppm (B20a) and 727ppm (B10) to 816ppm (B20b), likely due to poorer combustion caused by biodiesel's higher viscosity. Conversely, CO₂ emissions decreased, with B20a and B20b showing lower values compared to their respective base blends. Overall, B20a emerged as the best biodiesel blend for reducing NOx and CO₂ emissions, although it compromises slightly on CO emissions and engine runtime.

5.2 Recommendation

In this study, there are few recommendations to suggest for future study.

- i) For future study, it is recommended to investigate appropriate pre-treatment methods, such as acid esterification for WCO, to reduce FFAs prior to ultrasonic transesterification.
- ii) For future studies, utilize diesel engines equipped with sensors and systems designed to analyze fuel characteristics directly.

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APPENDICES

APPENDIX 1

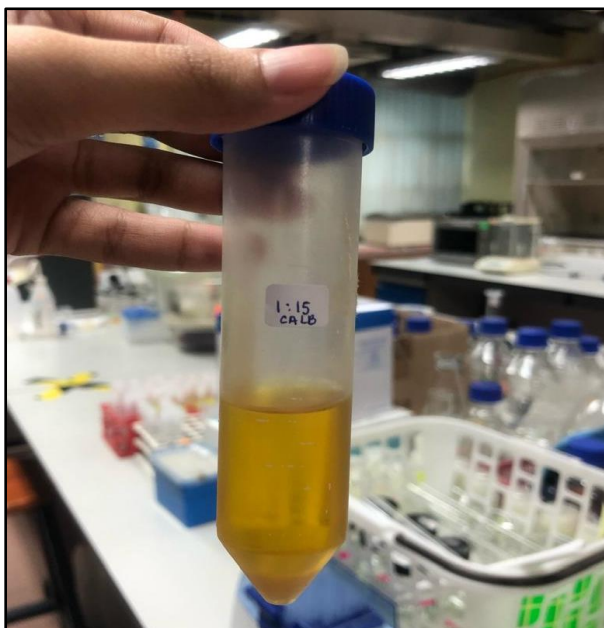
Comparative Study of Enzymatic Transesterification of WCO with Methyl Acetate Using Commercial Immobilized Lipases Between RMIM and CALB Under Ultrasonic Conditions



Experiment Set-up for Enzymatic Transesterification in Ultrasonic System



Commercial *Candida antarctica* Lipase B (CALB) Immobilized on Immobead 150
(Purchased From Sigma Aldrich Malaysia)



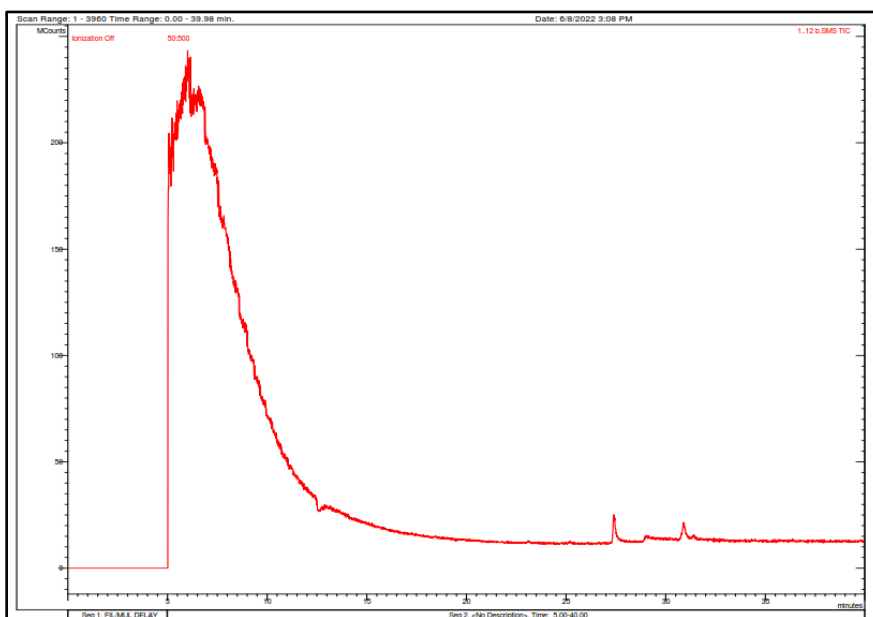
Biodiesel From Enzymatic Transesterification Reaction in Ultrasonic System



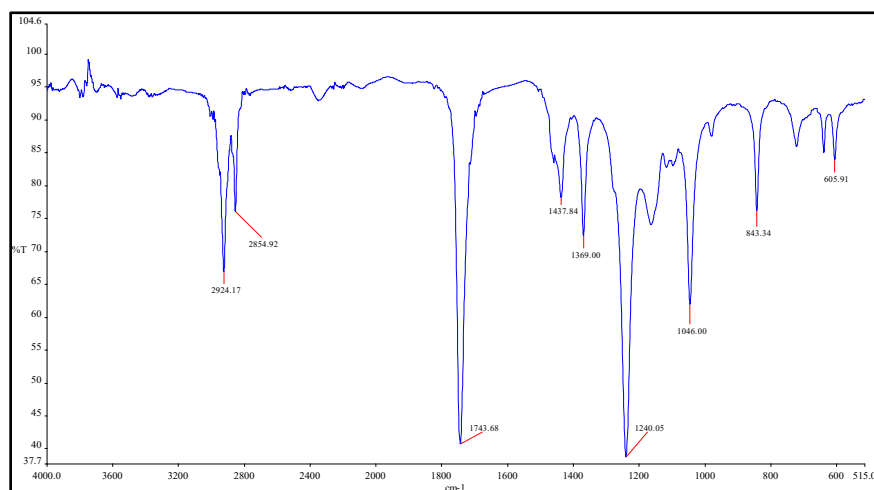
Weighing of 0.18g of Immobilized Lipases for the Transesterification Reaction
Experiment



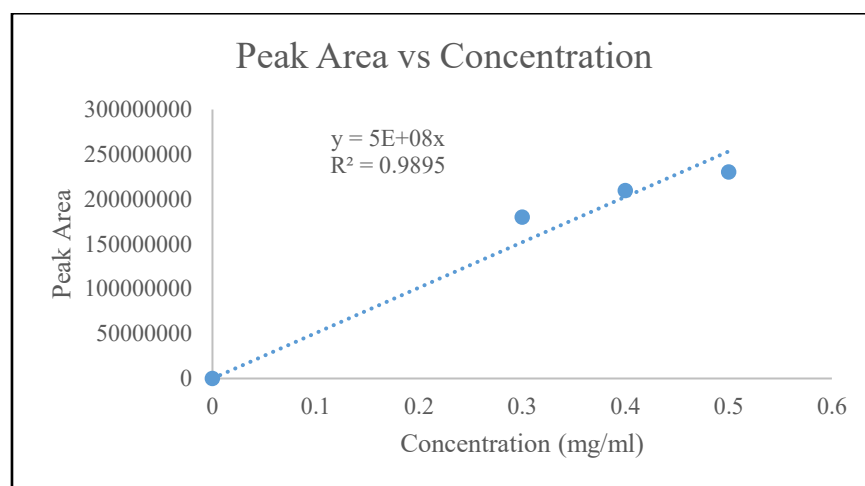
Three-neck Flask Set-up Containing WCO, Methyl Acetate, Lipase, Prepared for the Transesterification Reaction Experiment.



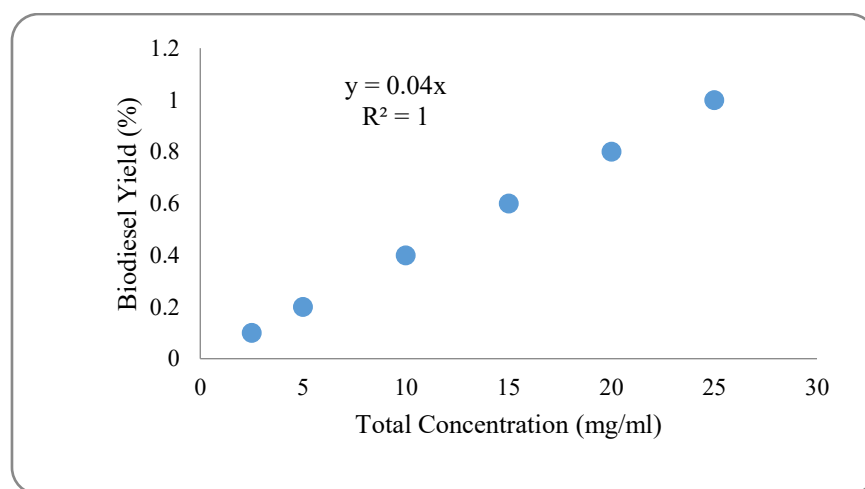
Chromatogram of Biodiesel Produced at 1:12 Molar Ratio of WCO to Methyl Acetate



FTIR Spectrum of Biodiesel Produced from (WCO) at 1:12 Molar Ratio of WCO with Methyl Acetate



Example of Calibration Curve for One FAME (Methyl Undecanoate)



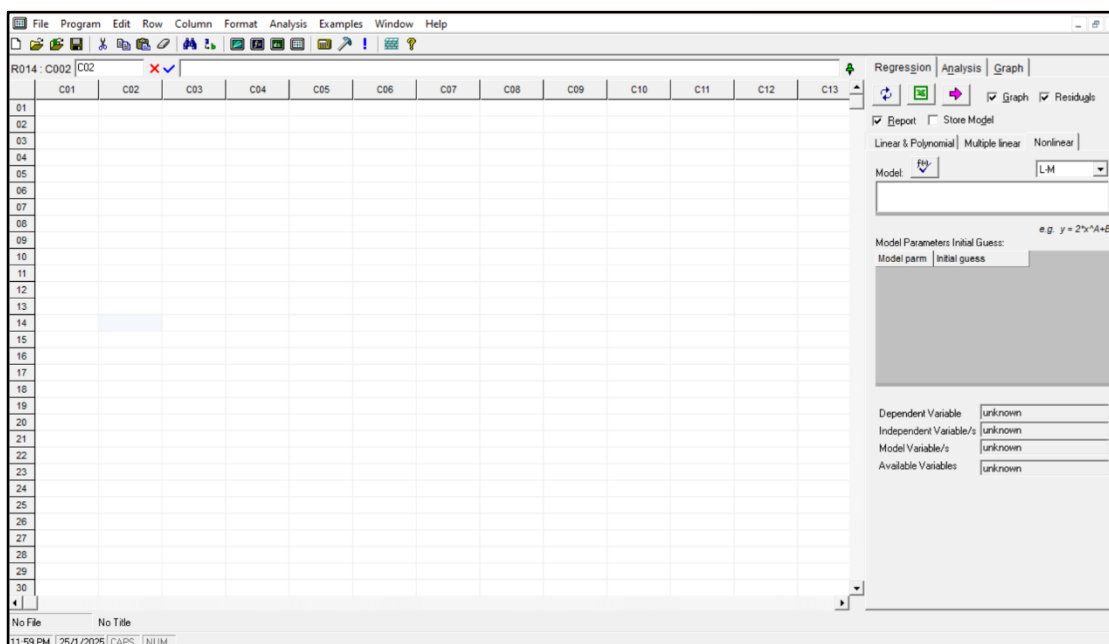
Calibration Curve Standard (C₄_C₂₄) for Total All FAME

APPENDIX 2

Kinetic Study for Enzymatic Transesterification of WCO With Methyl Acetate



Experimental Set-up for Initial Reaction Rate Measurement (0–30 minutes) in Biodiesel Synthesis Using Immobilized CALB



Polymath 6.10 Software

POLYMATH Report
Nonlinear Regression (L-M)

14-Dec-2024

Model: $V_i = (V_{max} \cdot TG \cdot A) / (K_m TG \cdot A \cdot (1 + A/K_i) + K_m A \cdot TG + TG \cdot A)$

Variable	Initial guess	Value	95% confidence
Vmax	0.00022	2.63E-05	2.32E-06
KmTG	0.0067	0.004961	0.0017019
Ki	0.042	0.0571274	0.019882
KmA	2.212	0.1784396	0.0894356

Nonlinear regression settings

Max # iterations = 64

Precision

R ²	0.994555
R ² adj	0.9782201
Rmsd	1.651E-07
Variance	6.812E-13

General

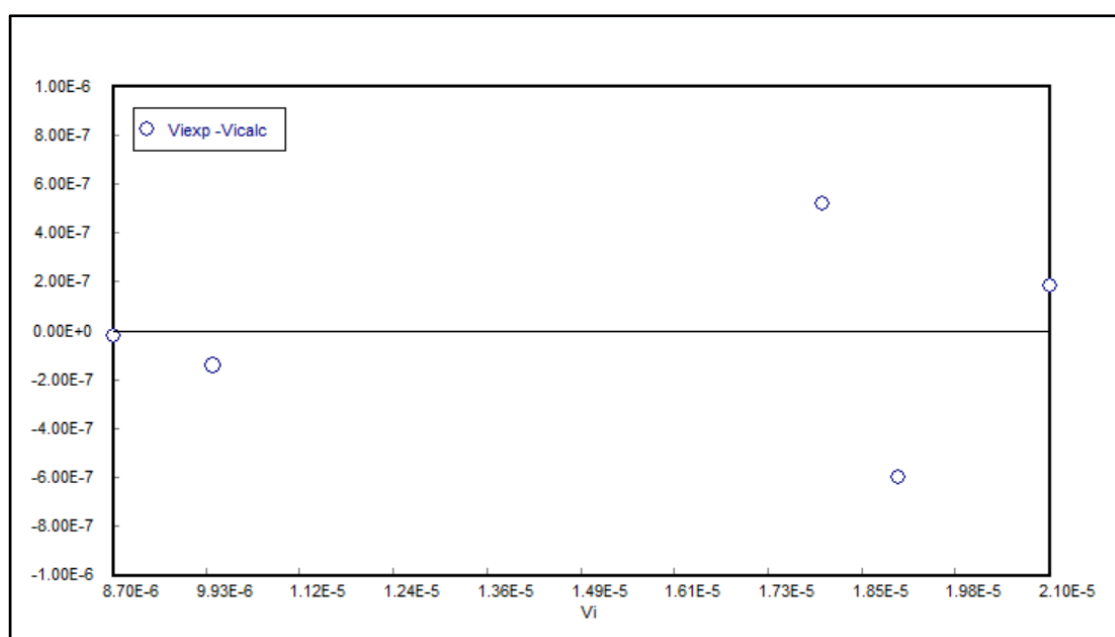
Sample size	5
Model vars	4
Indep vars	2
Iterations	11

Source data points and calculated data points

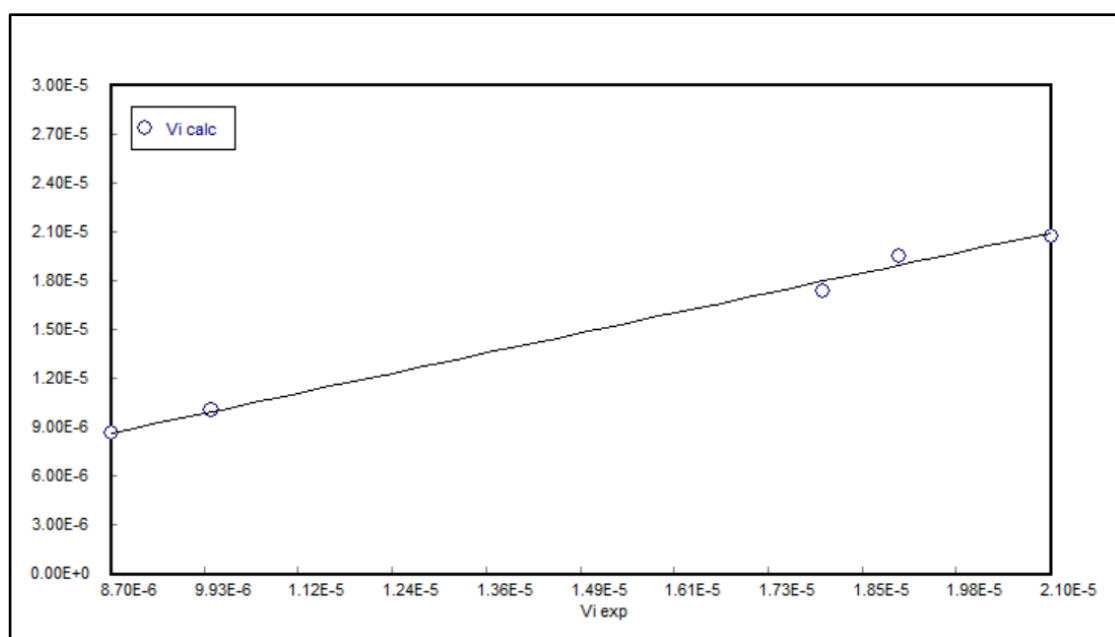
	TG	A	Vi	Vi calc	Delta Vi
1	1.0315	0.089	0.0000087	8.716E-06	-1.626E-08
2	0.9305	1.3118	0.000021	2.081E-05	1.902E-07
3	0.8291	2.5404	0.000019	1.959E-05	-5.924E-07
4	0.7271	3.7747	0.000018	1.748E-05	5.247E-07
5	0.4185	7.5129	0.00001	1.014E-05	-1.362E-07

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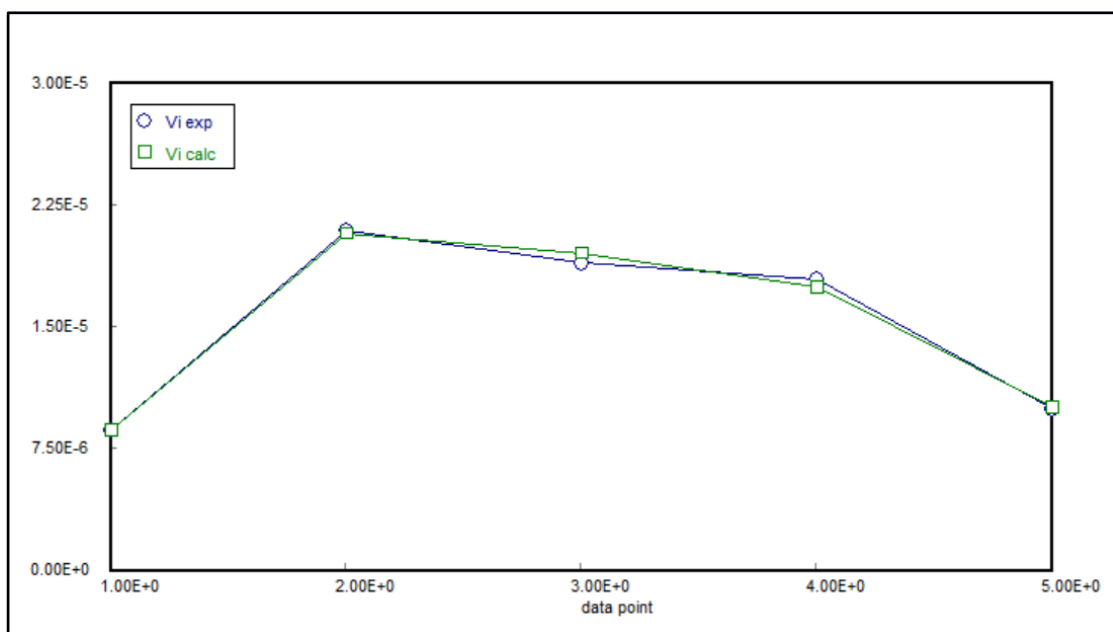
14/12/2024



Nonlinear Residual



Nonlinear Regression



Nonlinear Graph

APPENDIX 3

Biodiesel Blends Result From Diesel Engine

B20b Site: <#00000001> 20ml	B20b Site: <#00000001> 50ml	B20b Site: <#00000001> 20ml
***** * MRU VARIOPLUS * * 061 672 * *****	***** * MRU VARIOPLUS * * 061 672 * *****	***** * MRU VARIOPLUS * * 061 672 * *****
16.05.2023 07:11	16.05.2023 07:19	16.05.2023 07:26
Programm 1	Programm 1	Programm 1
T-gas 97.3 °C T-air 47.0 °C Dewpoint - O2 18.07 % CO2 1.70 % Losses ncw 0.0 % Eff. ncw 100.0 % CO 203 ppm CO 1128 mg/m³ Exc.Air 7.30 Draft -0.00 hPa NO 62 ppm NO 82 mg/m³ NOx 144 ppm NOx 276 mg/m³ NO2 83 ppm Diff.press. -0 Pa SO2 0 ppm SO2 0 mg/m³ H2S 0 ppm CH4 0.000 % CH4 0 mg/m³ Flow rate 0.0 m/s Flow vol. 0.0 Nm³/s	T-gas 102.1 °C T-air 47.3 °C Dewpoint - O2 18.13 % CO2 1.67 % Losses ncw 0.0 % Eff. ncw 100.0 % CO 876 ppm CO 1094 mg/m³ Exc.Air 7.42 Draft 0.00 hPa NO 57 ppm NO 76 mg/m³ NOx 136 ppm NOx 280 mg/m³ NO2 79 ppm Diff.press. 0 Pa SO2 0 ppm SO2 0 mg/m³ H2S 0 ppm CH4 0.000 % CH4 0 mg/m³ Flow rate 0.0 m/s Flow vol. 0.0 Nm³/s	T-gas 102.4 °C T-air 47.4 °C Dewpoint - O2 18.16 % CO2 1.63 % Losses ncw 0.0 % Eff. ncw 100.0 % CO 848 ppm CO 1060 mg/m³ Exc.Air 7.49 Draft 0.00 hPa NO 57 ppm NO 77 mg/m³ NOx 134 ppm NOx 276 mg/m³ NO2 77 ppm Diff.press. 0 Pa SO2 0 ppm SO2 0 mg/m³ H2S 0 ppm CH4 0.000 % CH4 0 mg/m³ Flow rate 0.0 m/s Flow vol. 0.0 Nm³/s

B20bx Site: <#00000001> 20ml	B20bx Site: <#00000001> 50ml	B20bx Site: <#00000001> 20ml
***** * MRU VARIOPLUS * * 061 672 * *****	***** * MRU VARIOPLUS * * 061 672 * *****	***** * MRU VARIOPLUS * * 061 672 * *****
16.05.2023 07:44	16.05.2023 07:52	16.05.2023 08:00
Programm 1	Programm 1	Programm 1
T-gas 93.8 °C T-air 47.3 °C Dewpoint - O2 18.07 % CO2 1.62 % Losses ncw 0.0 % Eff. ncw 100.0 % CO 216 ppm CO 1144 mg/m³ Exc.Air 7.26 Draft 0.01 hPa NO 56 ppm NO 75 mg/m³ NOx 134 ppm NOx 276 mg/m³ NO2 78 ppm Diff.press. 1 Pa SO2 0 ppm SO2 0 mg/m³ H2S 0 ppm CH4 0.000 % CH4 0 mg/m³ Flow rate 1.5 m/s Flow vol. 0.0 Nm³/s	T-gas 101.4 °C T-air 47.3 °C Dewpoint - O2 18.16 % CO2 1.53 % Losses ncw 0.0 % Eff. ncw 100.0 % CO 775 ppm CO 968 mg/m³ Exc.Air 7.47 Draft 0.01 hPa NO 62 ppm NO 83 mg/m³ NOx 137 ppm NOx 281 mg/m³ NO2 75 ppm Diff.press. 1 Pa SO2 0 ppm SO2 0 mg/m³ H2S 0 ppm CH4 0.000 % CH4 0 mg/m³ Flow rate 1.8 m/s Flow vol. 0.0 Nm³/s	T-gas 102.7 °C T-air 47.5 °C Dewpoint - O2 18.16 % CO2 1.49 % Losses ncw 0.0 % Eff. ncw 100.0 % CO 740 ppm CO 924 mg/m³ Exc.Air 7.48 Draft 0.02 hPa NO 65 ppm NO 88 mg/m³ NOx 140 ppm NOx 287 mg/m³ NO2 75 ppm Diff.press. 2 Pa SO2 0 ppm SO2 0 mg/m³ H2S 0 ppm CH4 0.000 % CH4 0 mg/m³ Flow rate 1.9 m/s Flow vol. 0.0 Nm³/s

Gas Emissions of Biodiesel Blends with B7 (Industrial Fuel) and B20 (Commercial Fuel)

AUTHOR'S PROFILE



Nur Fatin Syafiqah Mohamad Nor graduated from Universiti Teknologi MARA with a Bachelor of Engineering (Hons) in Chemical Engineering and has completed her Master of Science (Chemical Engineering). Her master's thesis involves several aspects of biodiesel production and enzyme kinetics, focusing on the transesterification of waste cooking oil using immobilized lipases within an ultrasonic system. The study incorporates methods such as reaction kinetic modelling, Ping-Pong Bi-Bi mechanism analysis, and enzyme inhibition studies. The research also explores the characterization of biodiesel fuel properties and its emissions performance in diesel engines, particularly focusing on NO_x, CO, and CO₂ emissions.

LIST OF PUBLICATION

Article

N. F. S. Mohamad Nor, H. Veny, F. Hamzah, M. N. Muhd Rodhi, R.D. Kusumaningtyas, H. Prasetiawan, D. Hartanto, S. Sulaiman and R. A. Sazali, (2024). "Enzymatic Transesterification Using Different Immobilized Lipases and its Biodiesel Effect on Gas Emission," *Bulletin of Chemical Reaction Engineering and Catalysis*, 19(2), 265–274.

Proceeding

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Immobilized Lipases,” *2nd International Symposium of Reaction Engineering, Catalysis & Sustainable Energy*.

H. Veny, N.A. Mohd Yusoff, N.F.S. Mohamad Nor, F. Hamzah, M.N. Muhd Rodhi, R.D Kusumaningtyas, H. Prasetiawan and D. Hartanto, (2022). 6th International Coference of Chemical Engineering & Industrial Biotechnology.