MEALWORM (*TENEBRIO MOLITOR)* OIL AND ITS POTENTIAL AS BIODIESEL FEEDSTOCK OPTIMISED BY RESPONSE SURFACE METHODOLOGY

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by

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LIST OF ABBREVIATIONS

2FI	Two-factor interaction
AAS	Atomic absorption spectroscopy
ANOVA	Analysis of variance
AOCS	American Oil Chemists' Society
AR	Analytical reagent
ASTM	American Society of Testing and Materials
BSFL	Black soldier fly larvae
BLSS	Bioregenerative life support system
CCD	Central composite design
CO_2	Carbon dioxide
Df	Degree of freedom
DoE	Design of experiment
DSC	Differential scanning calorimetry
DTG	Differential Thermogravimetry
EIA	Energy Information Administration
EN	European Standards
FAEE	Fatty acid ethyl esters
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organisation
FFA	Free fatty acid
FID	Flame ionisation detector
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography

GC-MS	Gas chromatography-Mass spectrometry
GHG	Greenhouse gases
GPC	Gel permeation chromatography
HCl	Hydrochloric acid
IEA	International Energy Agency
KI	Potassium iodide
KOH	Potassium hydroxide
MPOB	Malaysian Palm Oil Board
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulphate
$Na_2S_2O_3$	Sodium thiosulphate
Na_3PO_4	Sodium triphosphate
No.	Number
OCED	Organisation for Economic Co-operation and Development
PE	Polyethylene
PHA	Polyhydroxyalkanoate
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl chloride
RSM	Response surface methodology
S/N	Signal-to-noise ratio
SFA	Saturated fatty acids
TG	Triglycerides
TGA	Thermogravimetric analysis

UFA	Unsaturated fatty acids
ZnO	Zinc oxide

LIST OF SYMBOLS AND UNITS

°C	Degree Celsius
cm^{-1}	Per centimetre
$g\ cm^{-3}$	Gram per cubic centimetre cube
g	Gram
g^{-1}	Per gram
mg	Milligram
ml	Millilitre
$mm^2\ s^{-1}$	Millimetre squared per second
N	Normality
R^2	Coefficient of determination

MINYAK *MEALWORM* (*TENEBRIO MOLITOR*) DAN POTENSINYA SEBAGAI STOK SUAPAN BIODIESEL YANG DIOPTIMUMKAN OLEH METODOLOGI PERMUKAAN RESPON

ABSTRAK

Permintaan keperluan tenaga global yang berkembang pesat telah mendorong penemuan alternatif yang berpontensi untuk penggantian sumber minyak yang tersedia secara komersial. Salah satu alternatif ialah serangga yang diketahui mempunyai kandungan lemak yang tinggi bersesuaian dengan sifatnya seperti tabiat pemakanan dan kebolehdapatan di seluruh dunia. Kajian ini berusaha untuk menemui potensi *mealworm* (*Tenebrio molitor*) sebagai stok suapan biodiesel bagi penghasilan biodiesel yang dioptimumkan oleh kaedah metodologi permukaan respon (RSM). *Mealworm* mempunyai kandungan minyak yang agak tinggi iaitu 37.54 ± 0.78%, dengan kandungan asid lemak bebas (FFA) yang tinggi iaitu 10.84 ± 0.005%. Asid lemak utama *mealworm* ialah asid oleik (30.37%), asid linoleik (25.07%), dan asid palmitik (19.54%). Pirolisis minyak *mealworm* dijalankan pada suhu bilik sehingga 750°C pada kadar pemanasan 10°C seminit di bawah gas nitrogen dan ia terurai hampir sepenuhnya selepas 470°C. Pra-rawatan pengesteran bermangkin asid telah dijalankan untuk mengurangkan kandungan FFA yang tinggi ke kepada tahap di bawah 1.0% menggunakan parameter optimum 5.8% w/w asid sulfurik sebagai mangkin, nisbah metanol kepada minyak 24:1, masa tindak balas 174 min dan suhu tindak balas 74°C. Minyak mealworm yang telah dirawati adalah sesuai untuk pentransesteran bermangkin alkali dalam proses penukaran trigliserida dalam minyak kepada asid lemak metil ester (FAME). Hasilan biodiesel pada tahap 96.98% diperolehi dengan penggunaan natrium trifosfat sebagai mangkin alkali dalam keadaan tindak balas yang optimum (jumlah mangkin 3.2% w/w, nisbah metanol kepada minyak 16:1, masa tindak balas 114 minit dan suhu tindak balas 65°C) dan sifat-sifat biodiesel yang diperolehi memenuhi kualiti spesifikasi piawai ASTM D6751 dan EN 14214.

MEALWORM (TENEBRIO MOLITOR) OIL AND ITS POTENTIAL AS BIODIESEL FEEDSTOCK OPTIMISED BY RESPONSE SURFACE METHODOLOGY

ABSTRACT

The thriving global energy demand has driven the need for potential alternatives to replace the commercially available oil resources. One of the alternatives is insects, which are known to have high-fat content depending on their behaviour, feeding patterns, and worldwide availability. This study strives to discover the potential of mealworm (*Tenebrio molitor*) to be a stand-in bioenergy resource and converting it into biodiesel by optimising the process using response surface methodology (RSM). Mealworm had a high oil content of 37.54 ± 0.78% with a high free fatty acid (FFA) content of 10.84 ± 0.005%. The primary fatty acids of mealworm oil were oleic acid (30.37%), linoleic acid (25.07%), and palmitic acid (19.54%). The pyrolysis of mealworm oil was done from room temperature to 750°C at a heating rate of 10°C per minute under a nitrogen gas atmosphere and it decomposed almost completely after 470°C. An acid-catalysed esterification pre-treatment was conducted to reduce the high level of FFA content to a range below 1% using the optimum parameter of 5.8% w/w sulphuric acid as catalyst, 24:1 methanol-to-oil ratio, 174 minutes reaction time at 74°C to be favourable for alkali-catalysed transesterification to take place and convert the triglycerides in the oil into fatty acid methyl esters (FAME). The biodiesel obtained at a yield of 96.98% using sodium triphosphate as base catalyst under the optimised reaction conditions (catalyst amount 3.2% w/w, methanol-to-oil ratio 16:1, reaction time 114 minutes and reaction temperature 65°C) and the properties were found to meet the ASTM D6751 and EN 14214 standard quality fuel specifications.

CHAPTER 1
INTRODUCTION

1.1 Background

The growing energy consumption with the population pressure has drastically accelerated fossil fuel depletion as well as greenhouse gases emissions (GHG). Renewable energy plays a crucial role in clean energy transitions and enabling in keeping the global average temperature increases below 1.5°C (IEA, 2022b). Biodiesel (or fatty acid mono-alkyl esters) is a type of promising renewable energy to replace fossil fuels for its higher combustion efficiency, lower GHG emission lifecycle, higher cetane number, higher flash point and higher biodegradability (Vignesh & Barik, 2019). According to the International Energy Agency (IEA, 2018), a prediction of 30% renewable energy will be accounted for bio-energy by 2023. Biodiesel production in the world majorly utilises edible oils as feedstocks, especially palm oil in tropical countries, soybean oil in the US and rapeseed oil in the EU. However, the aspects of pressing global food security issues (van Huis, 2012), environmental problems for plantations (Martindale & Trewavas, 2008), and price have affected the use of conventional plants as biodiesel feedstocks supply. Over the years, scientists have been finding ways to overcome the challenges by utilising non-edible vegetable oil, wastes such as animal fats and waste cooking oil, and oleaginous microorganisms (typically microalgae) as conventional biodiesel feedstock substituents (Adewale et al., 2015; Behera et al., 2019; Hanif et al., 2022; Karabulut et al., 2018).

Insects, a potential source of oil, have been drawing the attention of researchers worldwide due to the ease of culturing insects and the ability of the insects to accumulate fat by feeding on organic wastes (Berezina, 2017). Insects are cheaper to cultivate, require less space, and grow faster than other livestock (Rumpold & Schlüter, 2013). The fat content of insects differs between order, species or development stages in the range from 1.5% up to 77%. Therefore, the maximum fat content of these insects is much greater than the oil content of edible oil such as corn oil (4.5-4.8%) (H. Wang et al., 2010), soybean oil (18-24%) (Qiong. Li & Zhang, 2018), rapeseed oil (37.5-46.3%) (Ishaq et al., 2017), sunflower oil (46-50%) (Rauf et al., 2017), and palm oil (50-55%) (Kasemsumran et al., 2012). However, studies showed that their diet is the most significant factor for their fat content and composition, with the larval stage having higher fat content under normal circumstances.

Mealworm, the larval stage of the darkling beetle (*Tenebrio molitor*), has a range of fat content at 17.70-40.45% extracted from their dry body biomass based on body matter weight basis. By noticing the significant amount of oil content of mealworms, it is feasible for mealworm oil to be an alternative feedstock of biodiesel production. However, insect oil has a high acid value, indicating high free fatty acid (FFA) content. A two-step process comprised acid-catalysed esterification and base-catalysed transesterification was required to fully convert the mealworm oil into biodiesel. The first step is to lower the FFA content to prevent saponification (El-Mashad et al., 2008). Saponifiaction will reduce the fatty acid methyl esters (FAME) content produced if the oil transesterified directly (Boey et al., 2012). The mixture was separated, and the unreacted triglycerides were then converted into FAME in the second step.

To date, no study has reported the optimisation of biodiesel production using mealworm oil. A range of optimum pre-treatment and biodiesel production conditions can be tailored by employing response surface methodology (RSM). This method is conducted to avoid neglecting each process factor's interactive effect compared with the conventional sequential optimisation method, minimising error to prevent numerous repetitive experiments. It generally provides insights into interactive effects between defined factors within the design space to determine the optimum condition of the required process. In RSM, an experimental design is usually incorporated with several independent factors to generate the design matrix to optimise the esterification and transesterification reactions involved in the pre-treatment and biodiesel production, respectively. In order to affirm the biodiesel produced is qualified according to the worldwide standards (typically EN and ASTM), characterisation with adaptation of methods in standards is usually performed to determine its fuel properties.

1.2 Overview of thesis

This thesis comprises five chapters which constitute the study background, experimental methodologies, and research findings. Chapter 1 gives a brief insight into the current biodiesel trend and the introduction of mealworms and highlights the potential of mealworms as the feedstock for biodiesel production, using RSM as an optimisation method.

Chapter 2 is a comprehensive review of the literature, which provides the background of mealworm as an insect species and its application in research. In addition, brief introduction on biodiesel is also being described in the following subsection. It is followed by the fundamental RSM and its approaches in optimisation.

In Chapter 3, the materials and methods used in characterising mealworm oil and optimising of pre-treatment process and the biodiesel production process, as well as the fuel properties of mealworm biodiesel, were listed and described to establish the detailed account of the procedures of each part of the process in completing the experimental part in this study.

Chapter 4 focuses on the experimental results and gives brief statements of actual results followed by the discussion, which includes comparison of the current study with the previous studies in the literature and evidence to support and out stand the findings. It breaks into 6 main sections, particularly the physiochemical properties, thermostability and fatty acid composition (FAC) of mealworm oil, followed by optimisation of the pre-treatment process and biodiesel production using RSM and ends with the fuel characteristics of mealworm biodiesel produced.

The last chapter concludes the research findings for this study. Recommendations for the future work of this research, such as further research on engine performance and as a raw material of lubricant, have also been provided to widen the prospect of mealworm oil in various applications.

1.3 Research objectives

This study's primary objective was to assess the feasibility of mealworm oil as a biodiesel feedstock with sulphuric acid (H_2SO_4) and sodium triphosphate (Na_3PO_4) as the catalysts for the pre-treatment and biodiesel production, respectively. The thesis revolves around the characterisation of mealworm, the optimisation of biodiesel production, and the quality assessment on the produced biodiesel according to the standards. Consequently, the objectives of this research are particularly as follows:

a. To determine the physiochemical properties of the crude oil extracted from the mealworm

b. To study the optimisation of the effect of the reaction parameters such as the effect of catalyst amount, methanol-to-oil ratio, reaction time and temperature on the pre-treatment process, on FFA content and biodiesel production via the statistical modelling approach method (RSM)
c. To analyse the fuel quality of mealworm biodiesel in accordance with the ASTM and European quality standards on biodiesel

CHAPTER 2
LITERATURE REVIEW

2.1 Insects

Insects are becoming more demanding and popular as a research subject in various scientific studies around the world due to their high potential for both nutritional and commercial aspects applications (Manzano-Agugliaro et al., 2012). With an estimated 5.5 million species, they are the most divergent in the *Animalia* kingdom (Stork, 2018). They are notorious for their ease of mass cultivation with high reproducibility, short life cycle, high adaptivity in poor and damp living conditions (Zheng et al., 2013), and inexpensive (Adamski et al., 2019). In the aspect of being environmentally friendly, they require a much smaller land area as compared to conventional livestock (Oonincx & de Boer, 2012), and this leads to a reduction of feed-to-food competition (Makkar, 2018), lower greenhouse gases (GHGs) emission (van Huis & Oonincx, 2017), and capability to decompose organic waste into low cost and sustainable biomass (Manzano-Agugliaro et al., 2012).

Over time, insects have been a sustainable source of animal feed (Sogari et al., 2019). A few insect orders, including *Orthoptera*, *Coleoptera*, *Isoptera*, *Hymenoptera*, and a few aquatic *Hemiptera*, are utilised as food and feed. The most abundant order in the animal kingdom is *Coleoptera* (beetle), with almost a quarter of all described animal species inhabiting the majority of our earth's environments (Stork, 2018). Insects have been part of human nutrition for alternative proteins and lipids, especially in Africa, Asia, and Latin America (Bodenheimer, 1952). They have similar protein content to fish, birds, mammals, and other vertebrates (Manzano-Agugliaro et al., 2012).

However, insects have a higher oil content than oil extracted from common edible oil crops such as corn (4.5-4.8%) (H. Wang et al., 2010), soybean (18-24%) (Qiong. Li & Zhang, 2018), rapeseed (37.5-46.3%) (Ishaq et al., 2017), sunflower (46-50%) (Rauf et al., 2017), and palm (50-55%) (Kasemsumran et al., 2012). The extracted mealworm oil is a non-edible by-product of the defatting process that can be used for various purposes. The oil can be extracted using different solvent extraction methods from the insects' dry biomass and generally with organic solvent due to its non-polar property—methods like Soxhlet extraction, Bligh & Dyer method, percolation, and maceration. The high-fat content is closely related to their propensity to accumulate fat by feeding on organic wastes (Berezina, 2017).

During the preadult stage development of insects (larva, pupa, or nymph), their body is capable of building metabolic reserves (Leeds, 1955; Tauber et al., 1986) that it can be used when it is not feeding, such as during metamorphosis, diapause, or other times of dormancy. During the metamorphosis process, they employ a fat body (a particular nutrient storage system) (Arrese & Soulages, 2010). Due to its role in intermediate metabolism, insects' fat stores energy and nutrients for later use. Insects accumulate fat reserves during their larval and adult stages, and this fat storage capacity boosts the insects' usefulness as a lipid alternative. However, studies have shown that their nutrition is the most crucial factor in determining their fat level and composition, with larval stage fat content higher than usual (Manzano-Agugliaro et al., 2012).

2.2 Mealworm (*Tenebrio molitor*)

Mealworm beetle, *Tenebrio molitor,* is a member of the detritivorous beetle family *Tenebrionidae*, the seventh-most diverse *Coleoptera* taxon with over 20,000 species in

2,200 genera (Z. Q. Zhang, 2011). Similar to other holometabolous insects, the mealworm beetle undergoes complete metamorphosis with four stages comprised egg, larva (mealworm), pupa, and adult in its entire life cycle as shown in Figure 2.1.

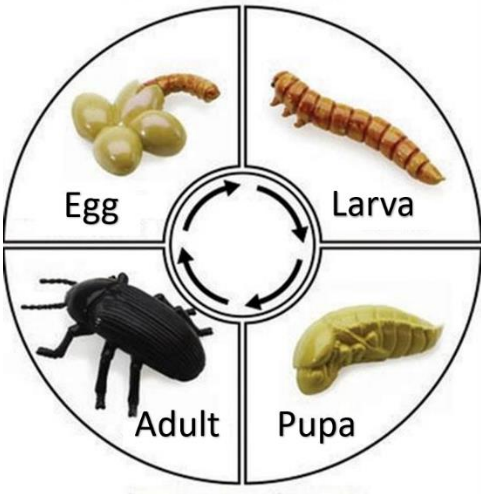


Figure 2.1 Life cycle of *Tenebrio molitor*

Source: Bradshaw, A. (2021). *How to raise mealworms for chickens*. [Photograph of Mealworm Life Cycle]; My Homestead Life. https://myhomesteadlife.com/how-to-raise-mealworms-for-chickens/

Each of the females of this species can produce an average of 500 eggs in every reproductive cycle. It remains in the larval stage for up to 3 months before transforming into a pupa. A mature larva has an average body length of 25-35mm (Alves et al., 2016). The larvae are typically treated as food and feed and are available in the insect market and aquatic pet stores worldwide. Mealworms can be mass cultivated easily by feeding wheat bran, oats, or grain with fruits and vegetables as their water source (Y. Yang et al., 2015). The feed conversion efficiency of mealworms enables their culture to be conducted more environmentally friendly than conventional livestock (Oonincx et al., 2015). The rearing can achieve a similar amount of nutrients and supports their use as a protein source with an optimal diet (Grau et al., 2017).

Due to its malleability and adaptability, different applications of mealworms have been studied by numerous researchers. Mealworm has been found to consume decaying organic matter like animals and leaves and is able to decompose into biomass and CO_2 by the bacteria in their guts (Bożek et al., 2017). Among the applications are the use of mealworms as food and feed, Styrofoam decomposing agent (Y. Yang et al., 2015), and biological recovery of polyhydroxyalkanoate (PHA) (Murugan et al., 2016).

2.2.1 Mealworm as a plastic decomposing agent

Interestingly, researchers found that mealworm can be fed petroleum-based synthetic plastics like polystyrene (PS), polyvinyl chloride (PVC), polyethylene (PE), and polypropylene (PP) and has the capability to degrade the plastics (Bozek et al., 2017; Fazal et al., 2019; Yang et al., 2015) into CO_2, biomass, and fecula (residue of egested plastic passed through the gut of mealworm). These synthetic plastics have severely threatened our environment over the years due to their high resistance to degradation. Yang et al. were the first to discover the gut microbiota inhabit in the mealworm's gut and were able to degrade PS and described it as an efficient bioreactor.

The feeding on the PS foam did not imply any adverse effect on their survival rate, just like the ordinary fed mealworms. Individually fed PS foam allowed them to survive for more than a month before morphing into pupae and becoming adult beetles (Nukmal et al., 2018; Y. Yang et al., 2015). It is believed that mealworms can be harmless on feeding on PS foam because of the detoxifying function of some microbiota in the mealworm gut (Genta et al., 2006). However, feeding these PS foams to mealworms caused nutritional deficiency, affecting their growth, development, and reproduction (Nukmal et al., 2018).

To improve biodegradation efficiency, it is recommended to have equal proportions of PS foam with regular feed to maintain appropriate growth with adequate nutrients and water (Fazal et al., 2019). The discovery of using mealworms in the depolymerisation and biodegradation of plastic waste has become an essential contribution to future applications in processing non-biodegradable and even biodegradable waste for our environment's benefit.

2.2.2 Mealworm as a thermoplastics biological recovery agent

In another respect, mealworms can be a potential polymer biological recovery agent. As described by Murugan et al. (2016), they identified that mealworm's digestive systems could recover a type of biodegradable thermoplastics known as PHA produced by certain bacteria. In the standard procedure, PHA extraction is conducted using an organic solvent like chloroform and dichloromethane, which requires a costly recycling process of the solvents. Other approaches using chemicals and solvents are also being conducted. Still, the drawbacks vary from the aspects of purity of the recovered PHA, recovery process cost, and sustainability of the recovery agents. To minimise using chemicals, solvents, and water, researchers utilized the animal model recovery process by feeding the chosen animal subject with the PHA-producing bacterial cells and consequently recovered PHA from the animal faeces (Ong et al., 2018).

Mealworms can decompose the bacterial cell by digestion and excrete the PHA granules. They discovered this ability preliminarily from the unchanged molecular weight of the PHA granules before and after the recovery process. The finding was further validated by various characterisations, including gas chromatography (GC), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), gel permeation chromatography (GPC), and rheology (Murugan et al., 2016). The advantages of this method are there is no harmful effect on the feeding using this bacterial cell to the mealworm and yet increasing the protein content, which is beneficial as food and feed, especially for aquaculture and poultries diets after the biological recovery process (Zainab-L et al., 2022; Zainab-L & Sudesh, 2019).

2.2.3 Mealworm as an alternative protein source

Apart from that, mealworms are also known for their protein-rich content and are generally used as livestock feed. Some studies show that the growth performance and digestibility of poultries fed mealworms as a substitute for their normal diet were comparable or even slightly enhanced (Bovera et al., 2016; De Marco et al., 2015; Hong et al., 2020). Mealworms are not only suitable for use as animal feed, but they may also be adequate for human nutrition. They have even been proposed as a bio-regenerative life support system (BLSS) for space missions due to their ability to process waste (L. Li et al., 2013). Mealworms may be found in a variety of different habitats across the world. In many countries, especially in Africa, Asia, and Latin America, mealworms are processed into food products or ingredients included in their daily food as an alternative source of protein (van Huis, 2012).

There is also research on separating protein from mealworms as a pure ingredient for food purposes (Choi et al., 2017; Kim et al., 2016; Son et al., 2020; Yi et al., 2013; Zhao et al., 2016). Research works are done to increase the protein concentration by expelling fat and chitin from mealworms (Kim et al., 2016), reduce agglomeration of extracted mealworm protein powder during the pulverisation process (Son et al., 2020), and, most importantly, the societal acceptance of consuming the extracted protein by incorporating in food rather than consuming the whole insect (Tan et al., 2017; Yi et al., 2013). Mealworm has to be defatted using organic solvent extraction or physically expelling the fat to acquire pure protein (Zhao et al., 2016). Consequently, the by-product (extracted oil) could be used as a potential feedstock for oil-based products.

2.2.4 Mealworm as potential oil source

As previously mentioned, mealworms have a high lipid content that makes them a promising source for oil-based products, such as biodiesel. The amount of lipids in mealworms varies depending on their developmental stage and diet (Zainab-L et al., 2022). Typically, lipid content increases as the mealworms mature, with the highest amount found in the last instar larvae (Kröncke et al., 2019).

Lipid secretion in mealworms is a complex process that is influenced by various factors. Nutritional status is one of the primary factors that affect lipid secretion. A study by van Broekhoven et al. (2015) demonstrated that larvae grown on a diet low in protein and high in carbohydrates had a lower fat content. In low-nutrition environments, larvae utilize their fat reserves for energy, resulting in lower fat content (Arrese & Soulages, 2009). Therefore, feeding mealworms a high-nutrition diet can help ensure a high fat content.

Moreover, the genetics of the mealworms can also play a role in lipid accumulation. Differences in lipid content have been observed between different strains of mealworms (Bragd, 2017). The stage of the life cycle can also affect lipid secretion, with the highest lipid accumulation occurring during the pupal stage (Morales-Ramos et al., 2016). Understanding these factors can help optimize mealworm rearing and lipid extraction to produce biodiesel.

2.3 Biodiesel

Meeting the growing demand for fuel has become one of the world's biggest problems, as the reservoir of economically viable fossil fuels will be depleted by 2050, according to experts (Campbell & Laherrre, 1998). The situation is likely to worsen with the world's population proliferating, and energy demand is estimated to increase to 45 Quadrillion Btu in 2050, a climb of 68% from 2019 (EIA, 2020). Combustion of fossil fuels increases CO_2 in the atmosphere, which causes global warming (Kasman & Duman, 2015; Mohsin et al., 2022), and a 6% (36.3 gigatons) growth of CO_2 is emitted compared to 2020, as observed in Figure 2.2. Biodiesel development as an alternative to petrodiesel in transportation could aid in energy, food security, and environmental and rural development (Babadi et al., 2022). Firstly, biodiesel is a vital aspect of the burgeoning bioeconomy and has the potential to replace fossil fuels. Secondly, biodiesel is a renewable energy generally produced from plant resources, animal fat, and waste cooking oil that can reduce greenhouse gas emissions when replacing fossil oil if sustainably managed (Venkatesh, 2022). Third, biodiesel feedstock crops are generally sourced from rural or disadvantaged farmers. It can provide employment to reduce poverty and improve farmers' lives (Nechad, 2022).

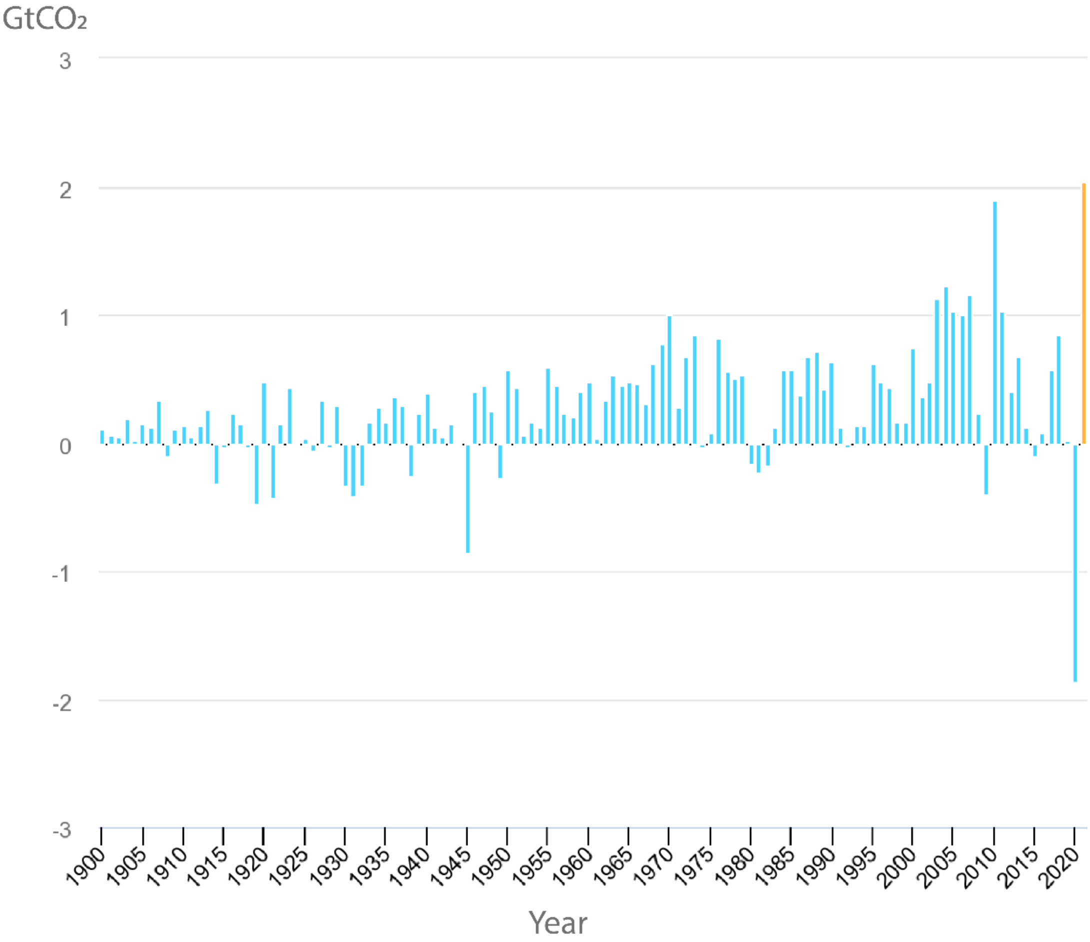


Figure 2.2 Annual change in CO_2 emissions from energy combustion and industrial processes, 1900-2021

Source: IEA. (2022a). *Annual change in CO_2 emissions from energy combustion and industrial processes, 1900-2021* (Licence: CC BY 4.0). IEA. https://www.iea.org/data-and-statistics/charts/annual-change-in-co2-emissions-from-energy-combustion-and-industrial-processes-1900-2021, Fig. 1, pg. 3.

Biodiesel is a non-toxic and renewable alternative fuel for diesel engines composed of mono-alkyl esters that can be generally produced from the triglycerides of diverse feedstocks such as edible and non-edible vegetable oil, animal fat, waste cooking oil via ester exchange process (transesterification) in the presence of short-chain alcohol (methanol, ethanol, propanol, and butanol) and a catalyst. The typical biodiesel types are FAME and fatty acid ethyl esters (FAEE) derived from the choice of methanol and ethanol, respectively. However, methanol is generally chosen due to its lower cost and higher reactivity than ethanol (Yusuf et al., 2011). They exhibit slight differences in fuel properties; for instance, the FAME has a lower viscosity and higher cloud and pour points than FAEE produced from the same feedstock (Bozbas, 2008).

Since 1992 and 1993, respectively, biodiesel has been manufactured on an industrial scale in the European Union (EU) and the United States of America (USA). EU currently produces and uses more biodiesel and renewable fuel than any other region in the globe. Approximately 13 million tonnes of biodiesel are produced each year at close to 200 plants located across the EU (EurObserv'ER, 2019). Global biodiesel output is anticipated to increase to 50 billion litres by 2030 due to rising petroleum costs and diesel fuel usage, as well as the promotion of numerous bio-energy fuels laws and consumption incentives. National policies that aim to aid farmers, lower greenhouse gas emissions, and/or boost energy self-sufficiency significantly impact the global biofuel industries (OECD/FAO, 2021b).

2.3.1 Biodiesel feedstocks

Biodiesel is traditionally derived from various kinds of renewable biomass feedstocks, such as vegetable oil, waste cooking oils, animal fats, and even algal oil. The feedstocks are classified into different categories in terms of the first generation (edible oleaginous crops), second generation (non-edible crops), and third generation (algal biomass, animal fats, and waste). Nearly 75% of biodiesel is derived from first-generation feedstock, vegetable oils (rapeseed oil makes up 20% of the total, soybean oil accounts for 25%, and palm oil contributes 30%) (OECD/FAO, 2021a). The types of oil differed from nation to nation based on the well-established estates and infrastructure for the edible oil crops to meet the up-scaling demand (Rincón et al., 2014). For instance, soybean oil is widely used by US and Argentina as biodiesel feedstock, followed by Brazil, Argentina, and Canada, whereas the EU employs rapeseed oil and palm oil favoured in tropical nations like Indonesia, Malaysia, Thailand, and Colombia (OECD/FAO, 2021a).

However, the expansion of the plantation of these edible oil crops may have adverse effects on the current food security in such shortages and price inflation in food production. Therefore, better biodiesel feedstocks that are environmentally friendly and prevent food insecurities but readily available at the same time should emerge.

Researchers have shifted their attention to non-edible feedstocks as a feasible solution to the problems with the first generation of feedstocks. Protecting the environment, reducing production costs, eliminating food insecurity, and reducing the need for arable land are the key benefits of second-generation feedstock. Second-generation feedstocks are generally lignocellulosic crops (Mohr & Raman, 2015), including Jatropha oil (Mustapha et al., 2022), Karanja oil (D. Kumar et al., 2018), rubber seed oil (Roschat et al., 2017), cottonseed oil (Onukwuli et al., 2017), and linseed oil (R. Kumar et al., 2013).

The types of non-edible plants as potential feedstocks vary from country and region. There are 35 kinds of feedstock that can be found in Pakistan (Rozina et al., 2017), 18 types in Africa (L. Yang et al., 2014), and 15 kinds in India (A. Kumar & Sharma, 2011). It can be foreseen the availability of enormous non-edible plant oil in the future to be a promising source for biodiesel feedstock (Silitonga et al., 2013; Y. Zhang et al., 2022). However, the lignin composition (H. Wang et al., 2017) leads to technical challenges, such as lower yield and high cost due to advanced technologies and infrastructure needed to convert it into biodiesel (Nigam & Singh, 2011). These restrictions inhibit the widespread adoption of second-generation biodiesel as a sustainable alternative to fossil fuels (Ganesan et al., 2020).

Third-generation feedstock, however, is a viable source for their cost-effective solution to minimise waste problems as they are mainly composed of microalgae, animal fat, and waste cooking oil (Y. Zhang et al., 2022). This generation has the advantage of less competition for oil crops and this feedstock in terms of land usage for cultivation (Saladini et al., 2016). Countries such as China, India, and the EU use waste cooking oil to produce biodiesel (OECD/FAO, 2021b). It results from cooking processes primarily in households, restaurants, food manufacturing sectors, and public events (games, festivals, concerts). It accounted for 22% of the total feedstock among the total feedstock in the EU back in 2021 and is predicted to surge to about 4% in 2022 (Flach et al., 2022).

The main spotlight in third-generation feedstocks is microalgae oil, which is sustainable in terms of high biomass yield, rapid growth rate, and ability in wastewater treatment (Maity et al., 2014; Yin et al., 2020). According to previous studies, the common microalgae genus employed to be a feasible feedstock is *Chlorella*, *Nannocholoropsis,* and *Spirulina*. However, the lipid productivity of microalgae is influenced by cultivation conditions, such as pH, nitrogen, salinity, and light intensity. The biomass will be reduced when there is an influx of high lipid amounts (Eloka-Eboka & Inambao, 2017). The total lipid of microalgae can range up to 54% dry cell weight depending on the species, resulting from their ability to accumulate lipids under autotrophic and heterotrophic conditions when nitrogen starvation-induced (Yaakob et al., 2021).

Animals derived fats like tallow, lard, and poultry fat that served as a biodiesel feedstock on an industrial scale can be obtained from the processing of cows, swine, and poultries, respectively. The rendering method usually extracts animal fats from animal meat processing facilities as a by-product. Other than that, biodiesel can be produced from the recovery of animal fat from the waste of the leather fleshing industry (Alptekin et al., 2012; Devaraj et al., 2018) and cascaded fish waste from fishmeal production for feed (Fadhil et al., 2017). Another good line of waste animal fat source is the insect fat (by-product) from the defatting process to produce biodiesel (Surendra et al., 2016). The defatting process was conducted as it can improve the extraction yield of insect protein. (Choi et al., 2017; Schiavone et al., 2017). This demonstrated that animal waste fat-based feedstock has advantages in economics, food security, and the environment over conventional edible vegetable oil feedstock (Adewale et al., 2015; Singh et al., 2020).

2.3.2 Biodiesel production

Generally, biodiesel is produced from an ester exchange method in which the triglycerides of the feedstocks are converted chemically into monoesters. The general ester exchange method can be achieved via transesterification, an alkali-catalysed chemical reaction involving the alkyl group of short-chain alcohol such as methanol, ethanol, and propanol with the extended and branched chain alkyl group of triglycerides to produce mono-alkyl esters and glycerol as a by-product. The usage of methanol is the widest because it is commercially available and inexpensive. The reaction scheme of transesterification is shown in Figure 2.3.

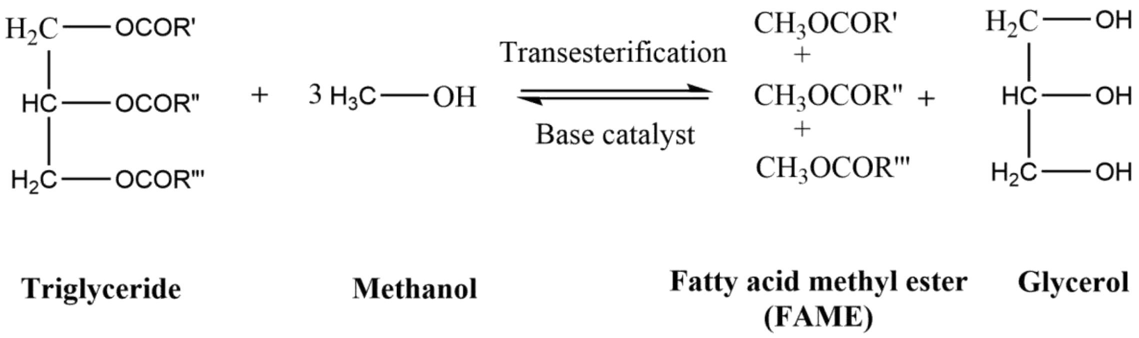


Figure 2.3 Transesterification process of triglyceride into FAME

However, feedstocks with more than 1% FFA content must be pre-treated with an esterification process using an acid catalyst before transesterifying into biodiesel. This is due to soap formation resulting from the neutralisation of FFA with the base catalyst during the transesterification process, which makes it hard to separate biodiesel and glycerine and reduces the ester content of the biodiesel. Strong acid catalysts such as H_2SO_4 are widely used for their high activity at low temperature and pressure (Nayab et al., 2022), cost-effectiveness (Malins et al., 2010), and ability to esterify FFA directly and transesterify the triglycerides (but at a prolonged rate) indirectly in the mealworm oil simultaneously (Marchetti & Errazu, 2008). FAME can be produced from the reaction between FFA and methanol, as shown in Figure 2.4.

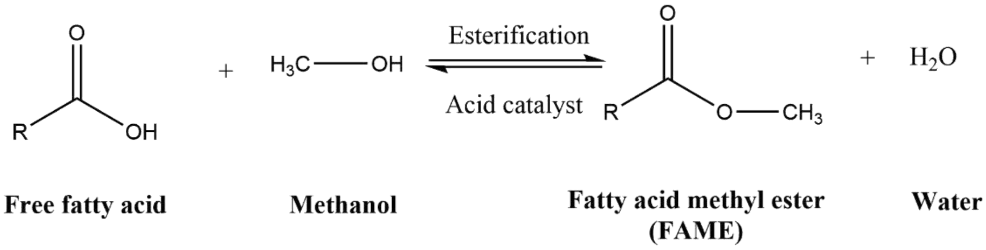


Figure 2.4 Esterification process of FFA into FAME

Both esterification and transesterification equations indicate that the processes are reversible. The biodiesel yield is directly influenced by a few relevant independent factors like catalyst amount, reaction temperature, duration of reaction, the molar ratio of alcohol to oil, and mixing speed. Hence, an optimal reaction parameter can ensure the reaction equilibrium is shifted to the right to produce biodiesel.

2.3.3 Insect oil as a feedstock for biodiesel production

Mealworm oil could serve as a potential source of feedstock for biodiesel due to their high lipid content. The fat content makes up around 33% of the dry matter of mealworms, the second-largest component after protein (Bragd, 2017). Studies from H. Wang et al. (2017) and Zheng et al. (2013) investigated the use of oil from mealworm as a biodiesel feedstock and found that it had a high content of fatty acid methyl esters, making it a promising source for biodiesel production. However, more research is necessary to optimize the rearing of mealworms and the extraction of their lipids for biodiesel production. It is worth noting that the nutritional composition of mealworms can differ depending on their developmental stage, with pupae having less lipid content than larvae (Morales-Ramos et al., 2016). As such, the life cycle stage of mealworms must be taken into consideration when utilizing them as a biodiesel feedstock. All in all, mealworms have the potential to be an eco-friendly and sustainable source of biodiesel feedstock.

There are a few different insect species other than mealworm that were studied by the researchers on their feasibility to be alternative biodiesel feedstocks. Among them are black soldier fly larvae (BSFL) (*Hermetia illucens*) (Jung et al., 2022), superworm

(*Zophobas morio*) (D. Leung et al., 2012), silkworm (*Bombyx mori*) (Nadanakumar et al., 2016) and housefly larvae (*Musca domestica*) (S. Yang et al., 2014).

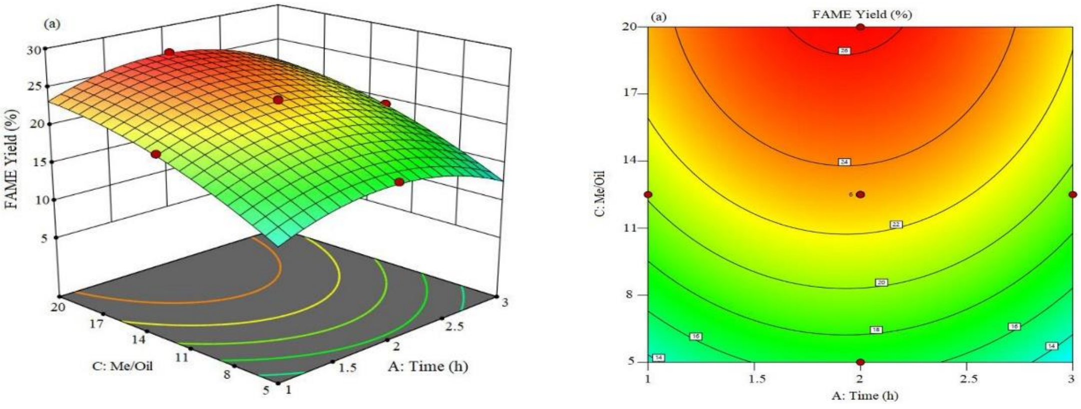
These insect oils shared a common characteristic which their biodiesel production was accomplished using a two steps process (acid and base catalysed esterification and transesterification) due to their high FFA content. The common acid catalyst being used is the H_2SO_4 to lower the FFA content in the oil for the next step. This pre-treatment step was necessary to ensure the transesterification could be conducted without the occurrence of saponification that will lower the ester content. The fuel properties of these insects' biodiesel will be compared and discussed with the mealworm biodiesel in this study in Section 4.4.

2.4 Response surface methodology (RSM)

Traced back to the 1950s, Box and Wilson (1951) first proposed the RSM in the context of chemical experimentation which involved mathematical and statistical techniques for both experimental design and analysis of the response surfaces. RSM refers to a collection of mathematical and statistical methods that are utilised to develop an appropriate functional relationship between a response of interest, y, and several associated independent variables (or input) that x_1, x_2….and x_n denotes. Thus, RSM is useful in the chemical industry's development, improvement, and optimisation processes (Dean et al., 2017).

Typically, a continuous quantitative scale is used to measure the response, and the RSM application often involves more than one response. The relationship between each input and the response can be demonstrated graphically; as such, it can be viewed in a contour plot (two-dimensional input-response plane) and/or a surface plot (threedimensional input-response plane). These plots are one of the best ways to demonstrate and comprehend how the response surface system operates. However, these plots are only for prediction, and if the same design was used to examine multiple sets of data, the system could change in small or big directions as each point on a plot has its standard error.

Figure 2.5 shows an example of a contour plot and surface plot of a response surface for two independent factors A (time) and C (methanol-to-oil ratio) from the study of Karimi & Saidi (2022):



Surface plot **Contour plot**

Figure 2.5 Example of contour plot and surface plot

Source: Karimi, S., & Saidi, M. (2022). Biodiesel production from *Azadirachta india* derived oil by electrolysis technique: Process optimization using response surface methodology (RSM). *Fuel Processing Technology*, *234*, 107337. https://doi.org/10.1016/j.fuproc.2022.107337, Fig. 7, pg. 10.

In most cases, such a relationship is unknown; however, the field of RSM served as an exploration of the space of a process to develop a suitable approximation between the response and process inputs. It also allows us to determine the levels of process inputs that produce desirable responses during the optimisation process. Normally, it can be determined using a low-degree polynomial model. A lack of fit test will be conducted for the design model to determine its adequation of response as a function of the chosen factors.

2.4.1 Design of experiment (DoE)

Particularly, the second order (quadratic) model is commonly used in RSM due to its flexibility in applying in various functional forms and efficiently approximating the true response surface. The general equation of the second-order model for the k factor can be represented as:

$$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k-1} \beta_{ij} x_i x_j$$

Where y=response for optimization combination of x= (x_1, x_2,x_k)

β_i=linear effect of the $_i$th factor.

β_{ii}=quadratic effect of the $_i$th factor

β_{ij}=interaction effect between the $_i$th and $_j$th factors

The response surface can be analysed to predict the response using the fitted second-order model when the lack of fit test is insignificant. A higher-order model experiment may be necessary if the second-order model significantly lacks fit. Curvature will present if the centre of the design is near the peak of the response surface, and thus multiple centre points are employed to compare the central mean response with the factorial points' mean response for a compelling lack of fit test.

2.4.2 Central composite design (CCD)

The central composite design (CCD) is one of the most often employed designs in quadratic models that Box and Wilson introduced for experiment design (Box & Wilson, 1951). It is an appropriate quality design for sequential experimentation and provides sufficient data for assessing the lack of fit without requiring excessive design points.

It employs a two-level factorial (resolution V design is required if it is fractional) with coded levels ±1 in conjunction with 2k axial points resulting from experiment runs involving factorial points, 2k axial points, and centre runs. The factorial point typically contains a maximum and a minimum level, expressed in the term 2^k, where k represents the number of factors in the design. Centre runs and axial points are essential to determine the existence of curvature (true replicate error) within the design system and effective approximation of pure quadratic terms, respectively. Figure 2.6 shows the CCD for k=2 and k=3 factors.

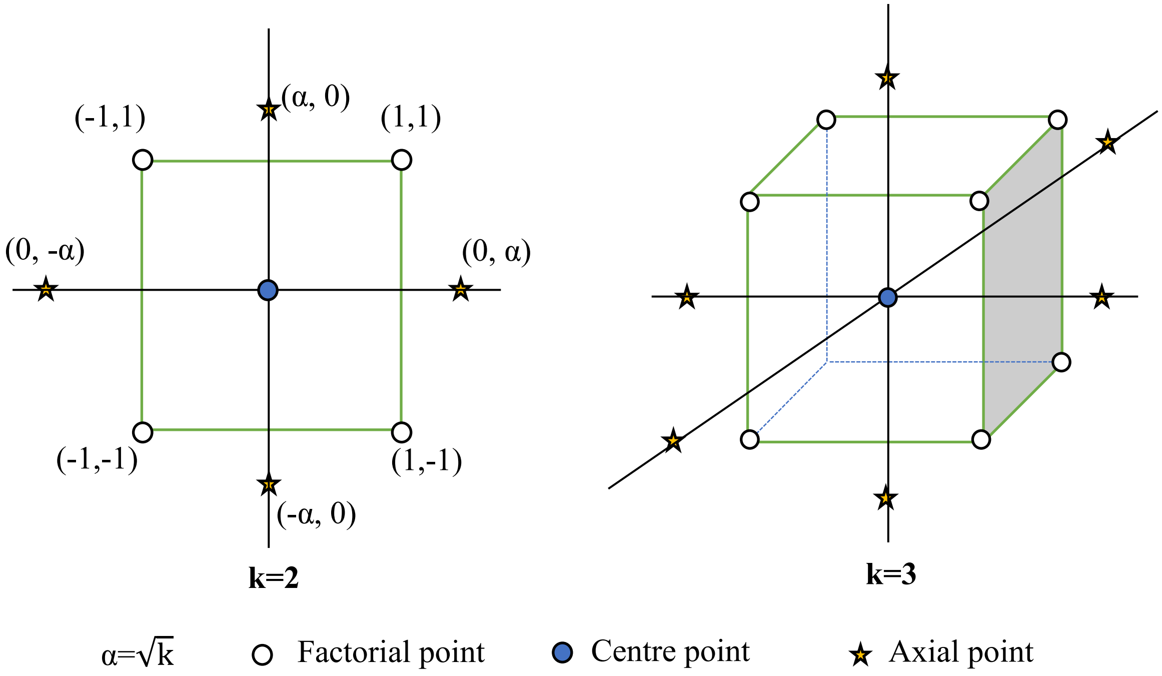


Figure 2.6 CCD for k=2 and k=3 factor