PRODUCTION, CHARACTERIZATION AND OPTIMIZATION OF GANODIESEL FROM THE BIOMASS OF *Ganoderma lucidum* PRODUCED IN AIR-L-SHAPED BIOREACTOR

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FACULTY OF SCIENCE
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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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PRODUCTION, CHARACTERIZATION AND OPTIMIZATION OF GANODIESEL FROM THE BIOMASS OF *Ganoderma lucidum* PRODUCED IN AIR-L-SHAPED BIOREACTOR

ABSTRACT

The escalating demand for alternative and sustainable energy sources, induced by the exploitation of fossil fuels and the surge in greenhouse gas emissions, has generated attention towards exploring rapidly-growing filamentous fungi as a potential bioenergy source. The objective of this study is to optimize *Ganoderma lucidum* production for enhanced biomass and lipid yields in submerged liquid fermentation. The optimization involved varying initial pH, glucose concentration, and agitation rate using response surface methodology (RSM) with central composite design (CCD). The results showed that glucose concentration and initial pH significantly influenced biomass production, while agitation rate had an insignificant effect. For total lipid production, all three factors (glucose concentration, initial medium pH, and agitation rate) were identified as significant factors. The optimized conditions for both responses (initial pH 6, 50 g/L glucose concentration, and 113 rpm) were validated in 500 mL shake flasks and a 3 L Air-L-Shaped Bioreactor (ALSB). In shake flasks, the biomass yield was 8.33 g/L and a lipid content of 2.17%, whereas the ALSB system yielded 5.32 g/L of biomass and 2.35% lipid. The *G. lucidum* mycelium lipid was extracted using solvent extraction, and the lipid profile was analyzed by gas chromatography-flame ionization detection. The main fatty acids identified included palmitic acid (C16:0) at 18.60%, stearic acid (C18:0) at 6.44%, oleic acid (C18:1) at 16.21%, and linoleic acid (C18:2) at 48.11%, which are recognized as major components of biodiesel. The *G. lucidum* mycelium lipid was converted into biodiesel (Ganodiesel) through acid-catalyzed transesterification, and subsequently evaluated in compliance with international biodiesel standards (ASTM D6751-08 and EN

14214). In addition, the findings suggested that the morphology of mycelial pellets varied across different fermentation conditions, resulting in distinct morphological characteristics for each condition. This study provides valuable insights into the potential of *G. lucidum* as an alternative biodiesel source by demonstrating optimized fermentation parameters and biodiesel production that comply with international standards.

Keywords: Optimization, fungi, submerged-liquid fermentation, lipid, biodiesel

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ABSTRAK

Kebutuhan yang meningkat untuk sumber tenaga alternatif dan mampan, yang dipacu oleh pengurangan bahan bakar fosil dan peningkatan pelepasan gas rumah hijau, telah menarik perhatian untuk meneroka kulat berfilamen yang cepat bertumbuh sebagai sumber bio tenaga yang berpotensi. Kajian ini bertujuan untuk mengoptimumkan penghasilan *Ganoderma lucidum* bagi hasil biojisim dan lipid yang ditingkatkan dalam fermentasi cecair. Pengoptimuman melibatkan variasi pH awal, kepekatan glukosa, dan kadar agitasi menggunakan "response surface methodology" (RSM) dengan reka bentuk komposit pusat (CCD). Keputusan menunjukkan bahawa kepekatan glukosa dan pH awal secara signifikan mempengaruhi penghasilan biojisim, manakala kadar agitasi tidak memberikan kesan yang signifikan. Untuk penghasilan lipid, ketiga-tiga faktor (kepekatan glukosa, pH medium awal, dan kadar agitasi) dikenal pasti sebagai faktor-faktor yang signifikan. Keadaan yang dioptimumkan untuk kedua-dua respons (pH awal 6, kepekatan glukosa 50 g/L, dan 113 rpm) disahkan dalam kelalang goncang 500 mL dan Air-L-Shaped Bioreactor (ALSB) 3 L. Dalam kelalang goncang, hasil biojisim adalah 8.33 g/L dengan kandungan lipid 2.17%, manakala sistem ALSB menghasilkan 5.32 g/L biojisim dan 2.35% lipid. Lipid miselium *G. lucidum* diekstrak menggunakan pengekstrakan pelarut, dan profil lipid dianalisis dengan menggunakan kromatografi gas-ionisasi nyala. Asid lemak utama yang dikenal pasti termasuk asid palmitik (C16:0) pada 18.60%, asid stearik (C18:0) pada 6.44%, asid oleik (C18:1) pada 16.21%, dan asid linoleik (C18:2) pada 48.11%, yang dikenali sebagai komponen utama biodiesel. Lipid miselium *G. lucidum* bertukar menjadi biodiesel (Ganodiesel) melalui pentransesteraan bermangkin asid, dan seterusnya dinilai mengikut piawaian biodiesel antarabangsa (ASTM D6751-08 dan EN 14214). Tambahan pula, hasil kajian ini mencadangkan bahawa morfologi pelet miselium berbeza-beza mengikut keadaan fermentasi yang berbeza, menghasilkan ciri-ciri morfologi yang berbeza untuk setiap keadaan. Kajian ini memberikan pandangan berharga tentang potensi *G. lucidum* sebagai sumber biodiesel alternatif dengan menunjukkan parameter fermentasi yang dioptimumkan dan pengeluaran biodiesel yang mematuhi piawaian antarabangsa.

Kata kunci: Pengoptimuman, kulat, fermentasi cecair, lipid, biodiesel

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LIST OF SYMBOLS AND ABBREVIATIONS

β	:	Beta
°C	:	Degree Celsius
µm	:	Micrometre
%	:	Percentage
ALSB	:	Air-L-Shaped Bioreactor
ANOVA	:	Analysis of variance
ASTM	:	American Society for Testing and Materials
BBD	:	Box-Behnken design
C	:	Carbon
CCD	:	Central composite design
cm	:	Centimetre
CO_2	:	Carbon dioxide
dm^3/h	:	Cubic decimetre per hour
DCW	:	Dry cell weight
EN	:	European standard
ENS	:	Endopolysaccharide
EPS	:	Exopolysaccharide
FAME	:	Fatty acid methyl ester
FAs	:	Fatty acids

g	:	Gram
g/L	:	Gram per litre
GC-FID	:	Gas chromatography-flame ionization detection
h	:	Hour
Hz	:	Hertz
K_2HPO_4	:	Dipotassium phosphate
kg/cm^3	:	Kilogram per cubic meter
KH_2PO_4	:	Potassium dihydrogen phosphate
kHz	:	Kilohertz
L	:	Litre
L/h	:	Litre per hour
MAE	:	Microwave-assisted extraction
m	:	Metre
mg	:	Milligram
mg KOH/g	:	Milligram of potassium hydroxide per gram
mg/g	:	Milligram per gram
$MgSO_4$	:	Magnesium sulphate
MHz	:	Megahertz
min	:	Minute
mL	:	Millilitre

mm	:	Millimetre
mm^2/s	:	Meter squared per second
mol	:	mole
MPa	:	Megapascal
MUFA	:	Monounsaturated fatty acid
NH_4Cl	:	Ammonium chloride
pA	:	Peak area
PDA	:	Potato Dextrose Agar
pH	:	Potential hydrogen
PUFA	:	Polyunsaturated fatty acid
rpm	:	Revolutions per minute
RSM	:	Response Surface Methodology
s	:	Second
$SCCO_2$	:	Supercritical carbon dioxide
SDGs	:	Sustainable Development Goals
SFA	:	Saturated fatty acid
SLF	:	Submerged-liquid fermentation
SSF	:	Solid-state fermentation
SVE	:	Solvent extraction
UAE	:	Ultrasonic-assisted extraction

UFA	:	Unsaturated fatty acid
v/v	:	Volume per volume
W	:	Watt
w/v	:	Weight per volume
wt%	:	Weight percentage

CHAPTER 1 INTRODUCTION

1.1 Research background

The swift growth of the population coupled with the need for lifestyle improvement has greatly increased the demand for water, food, and energy resources (Bamisile et al., 2020). Fossil fuels are contributing as the primary energy for the world's global energy demand and economy, particularly transportation fuel, which has a substantial environmental impact. Furthermore, fossil fuel resources are non-renewable and depleted on a daily basis (Shyamkishore et al., 2022). Thus, there are growing appeals for biodiesel as a feasible replacement to fossil fuels, given its benefits over other biofuels. Biodiesels are renewable, biodegradable, low emission of pollutants, low toxicity, eco-friendly, high lubricity and suitable for diesel engines with minimal mechanical modifications (Nguyen et al., 2023).

Recent research has suggested that various sources, including crops, waste frying oils and algal oil are viable feedstocks for biodiesel production. Nonetheless, the utilization of first-generation feedstocks from food crops poses issues related to competition for food production and changes in land use due to large-scale cultivation of edible crops for biodiesel purposes. Second-generation biodiesel and third-generation biodiesel, which employ non-edible crops and algae feedstocks, respectively, confront challenges such as environmental concerns associated with intensive water and fertilizer use, as well as obstacles related to scaling up and achieving cost competitiveness (Quah et al., 2019). Therefore, it is essential to investigate promising feedstock sources to facilitate the economical production of biodiesel without the need for arable land, thereby avoiding conflicts between food and energy production.

There has been less previous evidence for the use of fungi-based feedstock. Fungi can grow rapidly and are highly productive, often employed in biotechnology applications. It would be of special interest to study the effectiveness of fungi-based feedstocks as a new source of biodiesel (Hassan et al., 2019). *Ganoderma lucidum* is widely examined for its medicinal properties and bioactive compounds, and exploiting these diverse properties makes it an attractive fungus for lipid production research (Salvatore et al., 2020). Although some earlier research has studied the lipid content of *G. lucidum*, primarily in the fruiting body or spores, there remains a gap in knowledge regarding its mycelium lipids. *G. lucidum* is a xylotrophic basidial fungus that is capable of accumulating high level of lipids in the mycelium, the majority of which are triglycerides (Sharipova et al., 2016). Numerous research has shown that *G. lucidum* produces lipid with a fatty acid profile generally consisting of oleic acid (C18:1), palmitic acid (C16:0) and linoleic acid (C18:2), suggesting its potentiality for biodiesel production. Additionally, its simple, fast-growing nature and adaptability to different growth conditions enhance its potential for macroscale lipid production (Mohamad Jahis et al., 2022; Salvatore et al., 2020).

Furthermore, this research will apply response surface methodology (RSM) to optimize the fermentation parameters of *G. lucidum*, in terms of initial pH, agitation (rpm) and glucose concentration (g/L). Previous studies utilized RSM to analyze the optimal fermentation conditions for mycelium biomass and polysaccharide production (Supramani et al., 2019a). RSM is an effective method to determine the interaction and relationship that exists between the set of experimental factors and response variables compared to the conventional "one-factor-at-a-time" (OFAAT) method. RSM allows to evaluate the independent variables and analyze the significant and insignificant factors (Hassan et al., 2019).

This study aims to optimize the biomass and lipid production of *G. lucidum* mycelium for Ganodiesel production through RSM. This study will employ solvent extraction (SVE) techniques to extract lipids from *G. lucidum* mycelium biomass. The lipid profile of extracted lipid will be analyzed using gas-chromatography with flame ionization detection (GC-FID). After that, the obtained lipids will be subjected to transesterification for biodiesel conversion. The *G. lucidum* biodiesel (Ganodiesel) will be evaluated through several tests to ensure its compliance with biodiesel standards.

1.2 Problem statement, project objectives and hypothesis

1.2.1 Problem statement

The global energy crisis and environmental degradation caused by fossil fuel consumption have underscored the exploration of sustainable energy sources. Biodiesel has emerged as an alternative source, with feedstocks ranging from edible crops to non-edible feedstocks and algae. However, each of these feedstocks presents challenges, such as large-scale land use, competition with food crops and high production costs. Therefore, there is a critical need to identify an alternative feedstock that can produce biodiesel cost-effectively without these issues. There is a notable lack of research on the potential of mushroom biomass as a viable source for biodiesel production. In this study, *G. lucidum* will be cultivated using its mycelium in submerged liquid fermentation, rather than the fruiting bodies, which eliminates the need for arable land and addresses the challenges associated with current biodiesel feedstocks. Integrating response surface methodology (RSM) into this research can provide valuable insights into process optimization, contributing to the establishment of cost-effective biodiesel production.

1.2.2 Project objectives

1. To evaluate the influencing factors for the *Ganoderma lucidum* biomass and lipid production using response surface methodology (RSM) in shake flask and Air-L-Shaped Bioreactor (ALSB).
2. To characterize the fatty acid composition of *Ganoderma lucidum* lipid.
3. To produce biodiesel from *Ganoderma lucidum* lipid and analyze its specifications with international standards (ASTM D6751-08 and EN 14214).

1.2.3 Hypothesis

The optimum fermentation conditions such as initial pH, glucose concentration and agitation will have a significant effect on the *G. lucidum* biomass and lipid production. The major composition of fatty acids that exist in the *G. lucidum* lipid will be palmitic, oleic and linoleic acids. The properties of biodiesel derived from *G. lucidum* mycelial lipid will be able to comply with the biodiesel international standards.

1.3 Significance of study

1. This study introduces an innovative method to biodiesel production by utilizing *Ganoderma lucidum*, a landless feedstock that can be cultivated in a controlled environment without the need for arable land, specifically in a bioreactor.
2. Mushroom based-feedstock has received comparatively less attention in the biodiesel production domain. This study fills a research gap in the underexplored area of mushroom-based feedstock.
3. This study aligns with global goals for sustainable development (SDG 7-Affordable and clean energy; SDG 13-Climate action) by introducing a sustainable and potentially more environmentally friendly biodiesel feedstocks.

CHAPTER 2 LITERATURE REVIEW

2.1 *Ganoderma lucidum*

*Ganoderma lucidum***,** which is also referred to as "Lingzhi" or "Reishi" in Chinese. It is classified as a basidiomycete white rot macrofungus, belonging to the Fungi Kingdom. The fruiting bodies produced by *G. lucidum* have a flat kidney-shaped cap with a reddish-brown colour and are supported by the stipe. *G. lucidum* has been utilised widely in traditional herbal medicines in Asian countries, especially in China for over 2,000 years. *G. lucidum* is classified as one of the highest-grade "medicines" in traditional herbal medicines and is widely used today due to its substantial pharmacological significance (Sanodiya et al., 2009; Sharma et al., 2019; Zhang et al., 2013). The following stages: (1) spore dispersal, (2) hyphae formation, (3) mycelium colonisation, and (4) fruiting body formation constitute the reproduction system of *G. lucidum*. The mycelium exhibits rapid growth on agar plates, facilitating its cultivation in submerged liquid fermentation. Efficient growth of *G. lucidum* from its mycelium could be accomplished in a rapid time by optimizing parameters for submerged-liquid fermentation. Repeated-batch fermentation process under optimum fermentation parameters can achieve a better productivity of mycelium biomass in a shorter fermentation time (Hassan et al., 2019; Wan Mohtar et al., 2016).

The major bioactive components extracted from *G. lucidum*, particularly polysaccharides, triterpenes, sterols, proteins, and peptides have been remarkably studied in medicinal effects (Feng et al., 2014). Polysaccharides act as the prevalent bioactive metabolites in *G. lucidum*. The primary types of bioactive polysaccharides extracted from *G. lucidum* biomass are glucans, 1,3-beta-glucan, and beta (1,6)-D-glucan. Polysaccharides in relation to their structural properties have been found to possess numerous pharmacological properties, including antioxidant, anti-inflammatory, antimicrobial, anticancer, and immunomodulatory (Lu et al., 2020; Sharma et al., 2019).

G. lucidum mycelium biomass can be easily obtained from the method provided by Wan Mohtar et al. (2016) and further develop into valuable polysaccharides.

2.2 Predominant lipids composition of *Ganoderma lucidum*

So far, there is a general lack of research in biodiesel production derived from the basidial fungi-based feedstock. Although research on basidial fungi as a feedstock for biodiesel is still in its early phases, it has emerged as a promising and sustainable source. *G. lucidum* is a xylotrophic basidial fungus that is capable of accumulating high levels of lipids in the mycelium, the majority of which are triglycerides (Sharipova et al., 2016). Mohamad Jahis et al. (2022) demonstrated that *G. lucidum* mycelial biomass produced lipid extract containing predominantly oleic acid, palmitic acid and linoleic acid, suggesting its suitability for biodiesel production. Also, previous studies showed comparable lipid concentrations with a predominance of palmitic acid, linoleic acid and oleic acid are determined from Ganoderma species such as *Ganoderma austral* (Papaspyridi et al., 2012), *Ganoderma sinense* (Lv et al., 2012), *G. lucidum* and *Ganoderma applanatum* (Tokul-Olmez et al., 2018).

Several authors reported that mushroom-derived lipids are mainly composed of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, giving a high ratio of unsaturated to saturated fatty acids. Polyunsaturated linoleic acid (18:2), monounsaturated oleic acid (18:1), and saturated palmitic acid (16:0) have been identified as the primary fatty acids that exist in basidiomycetes. Table 2.1 depicts the major fatty acid proportions recognized in various mushroom strains. The content and composition of lipid in mushrooms can differ according to aspects like mushroom strains, cultivation parameters, solvents, as well as the extraction techniques (Tang et al., 2011). Mushroom-derived lipids contain bioactive compounds, particularly MUFA and PUFA, which can serve as the feedstock for producing biodiesel.

Table 2.1: Proportion of major fatty acids composition from several mushroom species (% of total FAs).

Species	Palmitic (C16:0)	Oleic (C18:1)	Linoleic (C18:2)	SFA	MUFA	PUFA	References
Agaricus bisporus	15.40	14.91	60.36	23.10	15.31	61.61	(Glamočlija et al., 2015)
	14.02	20.58	43.87	30.31	21.74	47.96	(Stojković et al., 2014)
Boletus edulis	21.60	31.10	33.80	33.40	31.10	35.50	(Kavishree et al., 2008)
	10.50	36.20	42.70	16.40	39.20	43.30	(Pedneault et al., 2008)
Cantharallus cibarius	20.21	13.57	59.79	20.21	17.92	59.79	(Yilmaz et al., 2013)
Ganoderma lucidum	18.20	68.40	3.19	26.40	70.30	3.19	(Salvatore et al., 2020)
	21.30	60.10	3.00	30.40	66.50	3.00	
	17.70	66.90	2.68	25.70	71.70	2.68	
Ganoderma applanatum	19.00	37.40	17.90	34.60	46.10	18.40	(Pedneault et al., 2008)
	18.30	22.50	28.80	21.87	-	-	(Abugri et al., 2016)
Pleurotus ostreatus	12.40	10.36	65.29	21.77	11.37	66.48	(Günç Ergönül et al., 2013)

*SFA: Saturated Fatty Acid; MUFA: Monounsaturated Fatty Acid; PUFA: Polyunsaturated Fatty Acid

2.3 *Ganoderma lucidum* cultivation techniques

Numerous studies have indicated that *G. lucidum* fruiting bodies and mycelia biomass consist of a wide range of bioactive compounds at high levels. The growing of fruiting bodies is a time-consuming step that demands large volumes of substrates and space, often resulting in inconsistent of sample quality. Thus, it is essential to prioritize mycelia cultivation, which offers a more precise and expedited approach compared to fruiting body cultivation (Bakratsas et al., 2021; Rathore et al., 2019). Mycelium cultivation can be broadly categorized into two techniques: submerged liquid fermentation and solid-state fermentation. Both techniques can be further subdivided into specific cultivation methods, depending on the equipment and conditions used. Submerged liquid fermentation can be conducted in bioreactors and Erlenmeyer flasks. Similarly, solid-state fermentation can be performed in bioreactors, bottles, bags, or other types of containers (Berovic et al., 2022).

2.3.1 Solid-State Fermentation (SSF)

Solid-state fermentation (SSF) refers to the process of cultivating and metabolizing microorganisms in the solid material beds containing sufficient moisture, where there is a continuous gas phase and limited unbound water in interparticle spaces. In SSF, the medium including water, nitrogen, phosphorus, carbohydrates, and sulphur is mainly bound within the solid particles, making it necessary for the applied microbial culture to possess the ability to extract these essential elements from the solid matrix. The process of solid-state fermentation is a heterogeneous phenomenon that involves solid, liquid, and gas phases (Berovic, 2019; Berovic et al., 2022). Different types of bioreactors are utilized for SSF, such as rotating drum bioreactors, packed-bed bioreactors, tray bioreactors, and stirred aerated beds bioreactors (Arora et al., 2018). SSF exhibits the convenience of utilizing a diverse range of substrates, including agricultural residues and waste material like rice husks, wheat bran, sugarcane bagasse, soybean straw and rice straw, without requiring extensive pre-treatment or enrichment. Additionally, SSF also produces less wastewater, making it an environmentally-friendly technique (Lizardi-Jiménez et al., 2017). However, obtaining an accurate measurement of biomass in SSF is challenging due to the close interconnected network between the mycelia and solid substrate, resulting in a compressed structure that hinders complete recovery of biomass (Manan & Webb, 2018).

2.3.2 Submerged Liquid Fermentation (SLF)

Submerged liquid fermentation (SLF) is an alternative technique used for growing various types of mushroom mycelium, including medicinal mushrooms. This method involves growing the fungi in a liquid medium that contains dissolved nutrients and is kept oxygenated through agitation, which enhances oxygen transfer and culture homogeneity. SLF can be conducted in various types of vessels, including static and agitated flasks, with forced aeration, and bioreactors (Balamurugan et al., 2021; Berovic & Podgornik, 2019). In SLF, there are multiple cultivation variables that can significantly influence the mycelium growth and biomass yield, such as temperature, initial pH, macronutrient and micronutrient sources, inoculum density, agitation, and aeration (Dudekula et al., 2020). By using submerged fermentation techniques, researchers can easily regulate the important process parameters, thus allowing for optimal growth and maximum nutrient utilization (Bakratsas et al., 2021). The mycelial biomass obtained using SSF or SLF from different mushroom strains is indicated in Table 2.2.

Table 2.2: The mycelial biomass obtained using SSF or SLF from different mushroom strains.

Mushroom species	Cultivation techniques	Mycelium biomass (DCW)	References
Pleurotus eryngii	SSF	108.50 - 422.49 mg/g 198.77 - 422.59 mg/g	(Melanouri et al., 2022) (Panagiota et al., 2019)
Pleurotus ostreatus	SSF	115.32 - 454.42 mg/g 247.70 - 340.32 mg/g	(Melanouri et al., 2022) (Panagiota et al., 2019)
Polyporus brumalis *Trametes suaveolens* *Trametes pavonia*	SSF	28 mg/g 28 mg/g 5.50 mg/g	(Simeng et al., 2015)
G. lucidum	SSF	100-150 mg/g	(Hu et al., 2022)
G. lucidum	SLF	3.12 g/L	(Hassan et al., 2019)
G. lucidum	SLF	5.19 g/L	(Supramani et al., 2019a)
G. lucidum	SLF	34.31 g/L	(Mohamad Jahis et al., 2022)
Ganoderma applanatum	SLF	17.51 g/L	(Balamurugan et al., 2021)
Ganoderma applanatum	SLF	13.34 g/L	(Diamantopoulou et al., 2014)
Ganoderma neo-japonicum	SLF	24.68 g/L	(Tan et al., 2015)
Lignosus rhinoceros	SLF	14.85 g/L	(Usuldin et al., 2023)
Pleurotus eryngii	SLF	10.85 g/L	(Chen et al., 2013)

*DCW: Dry cell weight

2.3.2.1 Fermentation parameters of *Ganoderma lucidum*

Several researchers have reported a correlation between the growth of *G. lucidum* mycelium and various factors, including the initial pH of medium, nutrient compositions, and agitation. Glucose serves as an essential carbon source for *G. lucidum* mycelial growth, typically present at levels ranging from 10 to 50 g/L. Moreover, acidic pH medium has been identified as more favourable for *G. lucidum* mycelial growth and synthesis of metabolites. The moderate agitation rate can effectively promote the nutrient mixing and oxygen transfer, thereby facilitating mycelium development and metabolites synthesis (Abdullah et al., 2020; Supramani et al., 2019a).

Fermentation modes such as batch fermentation and repeated-batch fermentation affected the exopolysaccharide and biomass productivity of *G. lucidum* (Wan Mohtar et al., 2016). The biomass production of *G. lucidum* is also affected by the fermentation broth factors, which are broth replacement ratio (BRR) and broth replacement time point (BRTP) (Abdullah et al. 2020). Broth replacement ratio is the process of removing a certain amount of fermentation broth and replacing with a fresh broth to enhance the growth cycle of *G. lucidum* mycelium. BRTP is to analyze the most suitable time frame for performing broth replacement. BRTP has categorized into 3 phases, which are the end of exponential stage, phases shifting stage, and stationary stage. For example, Abdullah et al. (2020) achieved the maximum *G. lucidum* biomass at 34.31 g/L through repeated-batch fermentation in 250 mL flask under optimal BRR and BRTP.

Moreover, Yuan et al. (2012) claimed that fermentation medium composition affected the exopolysaccharide production and mycelial growth of *G. lucidum*. Magnesium has a notable effect on mycelial growth. The optimal level of glucose in the fermentation broth promotes cell growth, resulting in higher production of exopolysaccharides. Hassan et al. (2019) determined that glucose concentration exerted a significant impact on mycelial biomass and exopolysaccharide productivity. The study identified that an optimal glucose level of 50 g/L resulted in the highest biomass and exopolysaccharide yields, which were 3.26 g/L and 1.96 g/L, respectively. Besides, agitation speed and initial pH influence the *G. lucidum* biomass production in submerged-liquid fermentation. Moreover, Supramani et al. (2019a) suggested that the maximum *G. lucidum* biomass can be achieved at 5.19 g/L with maintained conditions at pH 4, glucose concentration of 26.5 g/L and agitation at 100 rpm. In contrast, Chang et al. (2006) obtained a higher biomass yield at 18.70 g/L of glucose concentration, initial medium pH 6.5 and 160 rpm.

2.4 Lipid extraction

The process of lipid production from fungal mycelia involves multiple steps, including upstream processes of cell growth and lipid accumulation, followed by downstream processes of biomass harvesting and lipid extraction. The elaborate and rigid cell wall of mushrooms gives a hindrance to attaining full recovery of intracellular lipid, while the conventional solvent extraction method may not be adequate to extract the lipid effectively. The bioactive compounds are typically located within complex matrices in the mushrooms, making the extraction process challenging, often requiring extensive extraction time and more solvents (Roselló-Soto et al., 2016; Zainuddin et al., 2021).

Lipids are commonly classified into polar lipids and neutral lipids. Nonpolar or neutral lipids are types of lipid molecules that primarily serve as a storage form of energy in cells. Neutral lipids are uncharged and non-polar, which includes triglycerides, waxes, and sterol esters. These lipids are typically stored in structures such as lipid droplets within cells, where they reserve excess energy for future use. In contrast, polar lipids, including glycolipids, sphingolipids and phospholipids are involved in cell membrane structure, cell signalling, and lipid metabolism (Saini et al., 2021a).

Lipid extraction is a process of mass transfer that aims to separate lipid compounds from a sample to the extracting solvent by either disrupting the cells or allowing for penetration across the cell wall. Lipid is a type of organic molecules that are insoluble in water but can dissolve in organic solvents such as hexane, methanol, dichloromethane, and tetrahydrofuran. These solvents are commonly employed in lipid extraction to dissolve and isolate the lipid. The solvent phase is then subjected to solvent recovery procedures to remove the excessive solvent (Maddi, 2019). The selection of solvent for lipid recovery is according to the polarity of the desired lipids and their reciprocal solubility with the solvents. Polar solvents like ethanol or methanol are employed to disrupt the protein-lipid complexes, while nonpolar solvents like hexane or chloroform are used to solubilize neutral lipid triglycerides. Several studies have suggested that the blending of polar and non-polar solvents maximizes the total lipid extraction (Chen et al., 2018b; Mubarak et al., 2015). Ultrasonification, microwave-assisted or other chemical methods acting as cell disruption techniques are required to facilitate and efficiently extract lipid from fungi by disintegrating the rigid cell wall and releasing the lipid molecules (Chen et al., 2018b; Maddi, 2019). The comparison of lipid extraction techniques on lipid yield from several macrofungi species is indicated in Table 2.3.

Table 2.3: Comparison of lipid extraction techniques on lipid yield from several mushroom species.

Fungi species	Extraction techniques	Extraction Solvent	Operating conditions	Lipid yield (%Total lipid per biomass)	References
Boletus edulis	Supercritical CO_2 Soxhlet extraction	CO_2 Hexane	300 bar, 40 °C, 97.725 dm^3/h -	2.01 % 3.03 %	(Vidović et al., 2011)
Ganoderma lucidum	Supercritical CO_2	CO_2	30 MPa, 40 °C, 25 L/h	24.16 %	(Chen et al., 2012)
Ganoderma lucidum	Solvent extraction Soxhlet extraction UAE	Hexane Hexane Hexane	60 °C 60 °C 60 °C	14.57 - 20.36 % 9.44 - 18.80 % 4.30 - 7.50 %	(Mohamad Jahis et al., 2022)
Laetiporus sulphurous	MAE UAE	Hexane-Chloroform Hexane-Chloroform	25 W, 50 °C 40 °C	2.05 % 2.51 % 1.57 % 2.50 %	(Sinanoglou et al., 2015)
Lignosus rhinocerus	Soxhlet extraction	Hexane	60 °C	2.07 %	(Usuldin et al., 2023)
Lentinula edodes *Pleurotus eryngii* *Pleurotus ostratus*	UAE	Cyclohexane-isopropanol	300 W, 60 Hz	1.39 % 1.21 % 1.79%	(Saini et al., 2021b)
Boletus bainiugan *Laccaria laccata* *Tricholoma matsutake*	Folch method	Chloroform-methanol	-	16.56 % 5.24 % 10.17 %	(Yang et al., 2021)

2.4.1 Microwave Assisted Extraction (MAE)

Microwave-assisted extraction (MAE) has become increasingly popular owing to its high extraction efficiency and throughput. This technique utilizes a microwave generator to convey microwave energy to a sample containing a polarizable solvent and lipid-bearing compound (Mwaurah et al., 2020). Microwaves are referred to as the electromagnetic waves that range in frequency from 300 MHz to 300 GHz (Roselló-Soto et al., 2016). The microwave radiation can penetrate through the sample and react with polar compounds like water in the biomass, and heating the biomass evenly (Mubarak et al., 2015). As the sample is heated, the moisture present in the cells undergoes rapid evaporation, causing an increase in internal temperature and vapour pressure. Consequently, this pressure buildup can break down the cells, liberating the target intracellular compounds into the surrounding solvent. Generally, the optimal microwave frequency for MAE is around 2450 MHz (Zainuddin et al., 2021).

The factors of temperature, power, extraction time and solvent type on MAE can significantly impact the productivity and output of the extraction process (Heleno et al., 2016; Mwaurah et al., 2020). Carvalho et al. (2015) found that the total lipid content isolated from filamentous fungi *Mucor circinelloides* was increased with higher temperature and extraction time using microwave-assisted ethanol extraction. Other than that, Sinanoglou et al. (2015) conducted microwave-assisted extraction using hexane and chloroform to extract lipid from *Laetiporus sulphurous.* The results showed that n-hexane produced a substantially higher neutral lipid proportion than chloroform. Conversely, chloroform is more efficient in extracting polar lipid.

2.4.2 Ultrasound-Assisted Extraction (UAE)

Ultrasound-assisted extraction (UAE) has emerged as a frequently utilized methodology due to its superiority over traditional extraction techniques in the recovery of bioactive compounds. The high-frequency sound waves produced by ultrasonic create rapid changes in pressure in the solvent, leading to the emergence and implosion of cavitation bubbles. Generally, UAE is conducted within a frequency range of 20 to 50 kHz, which is considered effective for this technique (Mwaurah et al., 2020). When these bubbles collapse, high-intensity shock waves are generated to destroy the cellular membranes and promote the mass transfer of desired compounds, such as lipid, from the mushroom cells. The integration of ultrasonic waves in the extraction process can optimize the retrieval efficiency and kinetics by expanding the contact area between the sample and solvent, followed by disrupting the barriers to mass transfer within the sample through cavitation bubbles (Chen et al., 2018a; Roselló-Soto et al., 2016).

The following are the various factors affecting UAE-lipid extraction: (1) lipid extraction time, (2) temperature, (3) frequency, (4) biomass loading, and (5) solvent system (Jeevan Kumar et al., 2019). Kong et al. (2014) indicated that the extraction temperature, ethanol volume and time had significantly affected the total chlorophyll content extracted from *C. vulgaris* using UAE. It was observed that the yield of chlorophyll increased as the extraction temperature rose from 50 to 70 °C and extraction time extended from 0.5 to 1.5 hours. The optimization of lipid recovery obtained from *N. oculate* involves the utilization of 1000 W ultrasonic power, a 30-minute extraction period and 5% of dry weight biomass (Adam et al., 2012). Higher temperature and pressure intensify the generation of more violent cavitation bubbles collapse, subsequently causing increased shear stress and turbulence, leading to the degradation of extracellular matrix (Mwaurah et al., 2020).

2.4.3 Supercritical CO_2 extraction ($SCCO_2$)

Supercritical fluid extraction is a methodology for separating lipid and other bioactive compounds, involves the use of supercritical fluids at their vapour-liquid critical point. A supercritical fluid refers to any fluid that has been exposed to temperature and pressure conditions surpassing its critical point, causing the liquid and gas phases to become less distinct. At the critical point, the solvent exhibits both gas and liquid states, allowing it to have a high diffusivity and solvating power, which results in efficient mass transfer during extraction. Supercritical fluids are considered ideal extraction solvents because of their dependency on density for solvent efficacy, a controllable parameter through varying the extraction temperature and pressure. This technique allows to produce crude lipid without the need for a separate solvent removal step. Carbon dioxide (CO_2) is the most preferred supercritical solvent owing to its moderate critical pressure and temperature of approximately 7 MPa and 31 °C, respectively, which reduce the deterioration of thermally labile compounds during the extraction process (Roselló-Soto et al., 2016).

The performance of $SCCO_2$ extraction of fungi lipid is significantly influenced by pressure, temperature, and extraction time (Bogdan et al., 2014). In research by Vidović et al. (2011), the extraction yield of *Boletus edulis* lipid showed a significant increase when pressure and extraction time were increased from 100 to 300 bar and 1 to 3 hours, respectively. The lipid extraction yield of *Agaricus blazei* was observed to enhance with increasing pressure from 300 bar until the maximum value at 400 bar, with maintained temperature and flow rate. Higher pressure levels augment the density of supercritical carbon dioxide, which enhances its solvating capabilities. Conversely, the lipid yields enhanced with temperature elevation from 40 °C to 70 °C when the pressure and flow rate were held constant. The increase in temperature raises the vapour pressure of lipid, making them dissolve more readily in supercritical carbon dioxide (Coelho et al., 2005).

2.4.4 Solvent extraction

Solvent extraction is another method that has been commonly employed for lipid retrieval from oleaginous fungi. The two most conventional approaches to solvent extraction are Folch method and Bligh and Dyer method. The Folch method, published in 1957, utilizes a solvent combination of chloroform and methanol in a ratio of 2:1 v/v. On the other hand, Bligh and Dyer method, introduced in 1959 as a modification of the

Folch method, employs a solvent mixture of chloroform, methanol and water at a 2:2:1.8 v/v/v ratio (Breil et al., 2017; Saini et al., 2021a). These methods are based on a two-step extraction process utilizing both polar and non-polar solvents. The addition of water with subsequent steps of homogenization and centrifugation induces the formation of two distinct layers through phase separation. The upper layer consists of the polar solvent, while the bottom layer consists of the non-polar solvent, which contains the lipids (Tsirigka et al., 2023).

In recent studies, a solvent extraction process with modification has been demonstrated for extracting lipids from microorganisms. This solvent extraction method involves utilizing an organic solvent and dried biomass, subjecting the mixture to heat and agitation on a magnetic stirrer hot plate. Then, centrifugation or filtration is employed to separate the biomass, leaving the lipids dissolved in the solvent. The lipids are subsequently obtained by evaporating the excessive solvent through a rotary evaporator (Fattah et al., 2020; Shin et al., 2018). The method ensures better surface contact between biomass and solvent, improving the lipid extraction efficiency, and giving a higher lipid yield (Kiyani et al., 2023). Additionally, Mohamad Jahis et al. (2022) showed that the solvent extraction method (SVE) using hexane solvent, at a temperature of 60 °C and agitation speed of 200 rpm, resulted in a higher *G. lucidum* lipid yield compared to UAE and Soxhlet extraction.

2.5 Biodiesel

The utilization of fossil fuels has been integral to driving the global economy since the industrial revolution, supporting economic growth and industrial development for centuries. In 2020, fossil fuels continued to dominate the global energy landscape, accounting for about 84% of total primary energy consumption, with oil being the most widely used. These finite energy sources are estimated to remain major contributors to the world’s energy supply in forthcoming times (Feng et al., 2022). The depletion of fossil fuels and sustainability concerns have become significant challenges in the energy sector. Numerous studies have highlighted the limited availability of fossil fuels and the pressing need for sustainable energy sources to meet future energy demands. The depletion of fossil fuels poses challenges to both energy security and the environment, leading to environmental degradation and climate change (Yaqoob et al., 2021). Studies have extensively documented the adverse repercussions of fossil fuels combustion, including air pollution, greenhouse gas emissions, and alterations in climate patterns. Furthermore, the combustion of fossil fuels discharges contaminants such as carbon dioxide, nitrogen oxides and sulphur dioxide, contributing to air quality deterioration and respiratory diseases. Fossil fuel exploitation and processing also have detrimental effects on ecosystems, biodiversity, and water resources (Ogunkunle et al., 2021).

Biodiesel has been recognized as a promising and sustainable alternative energy source for emitting fewer greenhouse gases and air pollutants (Aljaafari et al., 2022). There is notable research in the field of biodiesel, focusing on various aspects such as production methods, feedstocks, environmental impacts, and applications. Recent biodiesel studies have concentrated on the advancement of biodiesel blends, including B5, B10, B20, and B100, with each number indicating the percentage of biodiesel combined with petroleum diesel. These blends have been utilized in various applications to enhance combustion characteristics and reduce emissions (Lamba et al., 2022). One notable application can be observed in the automotive sector, where biodiesel is employed as a fuel source for powering automobiles, agricultural machinery, and heavy construction equipment. Additionally, biodiesel-powered generators are utilized in the provision of backup power during instances of scarcity (Aljaafari et al., 2022).

Biodiesel is generally classified into (1) first-generation; (2) second-generation, and (3) third-generation biodiesel. First-generation biodiesels are made from edible oil materials such as soybean, rapeseed, palm, and sunflower. Second-generation biodiesels are made from non-edible oil feedstocks such as rubber seed, karanja, jatropha and waste cooking oil. On the other hand, algae and other microorganisms contribute to the third-generation biodiesel (Singh et al., 2014). Various countries around the world produce biodiesel feedstock from different sources, contributing to the global biodiesel industry. In the Philippines, coconut oil is a significant biodiesel feedstock, while *Jatropha curcas* oil is commonly utilized in India. Palm oil is the predominant source for biodiesel production in Indonesia, Thailand and Malaysia. In China, waste cooking oil serves as the major feedstock. The United States and Brazil predominantly rely on soybean oil, whereas rapeseed is the primary feedstock in Europe. This diversity of biodiesel feedstocks reflects local agricultural resources and industrial practices in different regions (Ifeanyi-Nze, 2022).

However, crop-based feedstocks have intensified environmental impacts and food versus fuel conflict. The extensive cultivation of food crops as feedstock for biodiesel production further burdens available agricultural land, impacting food production, thus exacerbating global food insecurity and economic imbalance (Abdul Hakim Shaah et al., 2021). For example, in Brazil, the expansion of soybean plantations has been driven by the constrained availability and imbalanced distribution of soybean products, which are allocated for meal, vegetable oil, and biodiesel production (Wilkinson et al., 2010). This surge in need for soybean oil has resulted in a significant expansion in soybean cultivation area, growing by 2.6 times from 13.4 million hectares in 2001 to approximately 30 million hectares in 2019, resulting in extensive deforestation (Rodrigues, 2021; Song et al., 2021). Similarly, deforestation and land conversion have occurred in Southeast Asia, particularly in Indonesia and Malaysia, for palm oil plantations aimed at biodiesel production. This has resulted in significant biodiversity loss and increased carbon emissions. Additionally, the extensive use of chemical fertilizer in these plantations has led to soil degradation (Papilo et al., 2022; Yasinta et al., 2021). Other than that, large-scale *Jatropha curcas* cultivation in India has resulted in land use conflicts, water scarcity, and social issues due to the displacement of food crops and local communities. Soil erosion and freshwater eutrophication are among the environmental concerns associated with fertilizers use in plantations areas (Gmünder et al., 2012). Therefore, there is a growing focus on exploring alternative feedstocks that do not interfere with food production while tackling environmental concerns, and optimizing efficiency and sustainability.

2.5.1 Conversion of lipid into biodiesel via transesterification

Biodiesel is generally referred to as fatty acid methyl esters (FAME) or B100, a fuel composed of mono-alkyl esters of long chain fatty acids. The biodiesel is required to comply with the requirements of ASTM D6751-08. The production of biodiesel occurs through a chemical process that involves reacting oil or fat, primarily composed of triglycerides, with an alcohol in the presence of catalyst to yield FAME and a by-product glycerol (Knothe, 2017). The overview of lipids conversion into biodiesel is illustrated in Figure 2.1.

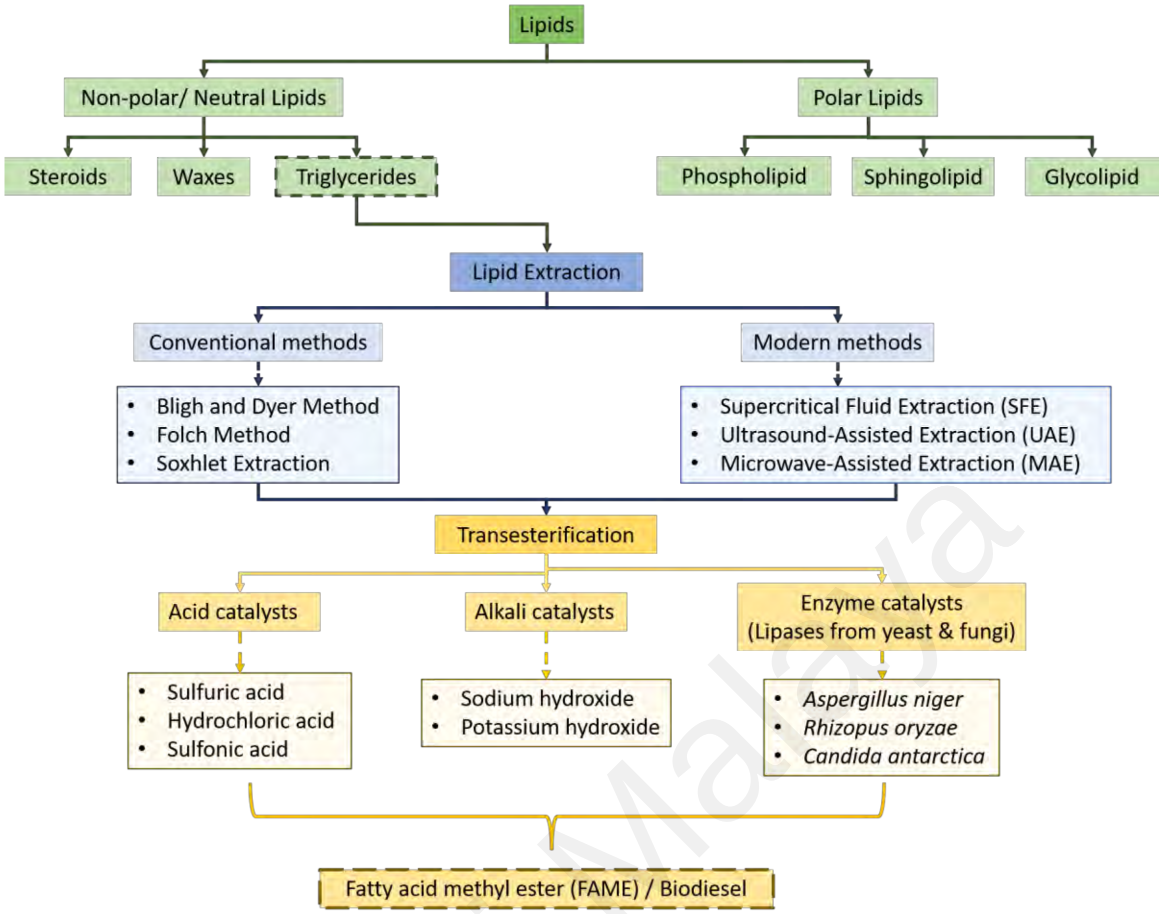

Figure 2.1: Overview of biodiesel production. (Figure by author)

Transesterification is a reversible chemical process that involves alcoholysis, where the alcohol can be displaced from an ester to shift the equilibrium towards the production of additional esters. The esters product of this reaction is known as biodiesel. Ethanol and methanol are the most widely chosen alcohols in transesterification for FAME synthesis (Baadhe et al., 2014; Mumtaz et al., 2017). Transesterification comprises a series of three consecutive reversible reactions. Initially, triglyceride (TG) reacts with alcohol to convert into diglyceride (DG) and a fatty acid methyl ester (FAME). Consequently, diglyceride (DG) is broken down to monoglyceride (MG), which is then converted into glycerol. Each reversible reaction yields a fatty acid methyl ester (FAME), as depicted in Figure 2.2 (Maheshwari et al., 2022; Mumtaz et al., 2017).

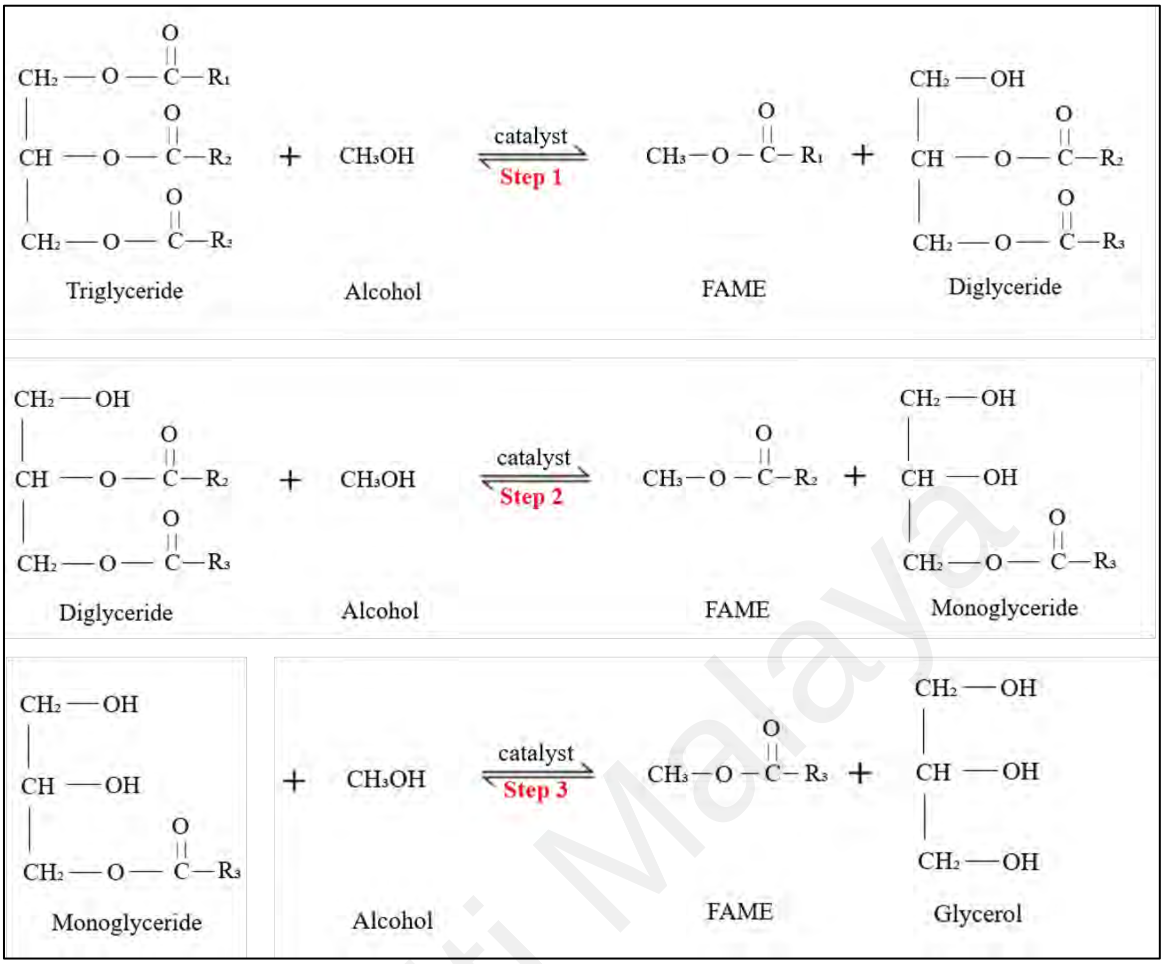

Figure 2.2: Overall transesterification reaction. (Figure by author)

Biodiesel production via transesterification can be achieved through chemical or enzymatic methods. Chemical transesterification reactions are classified as acid-catalyzed transesterification and base-catalyzed transesterification. Acid-catalyzed transesterification reactions favour the feedstock with higher free fatty acid content (FFA), thus giving a higher yield of FAME compared to alkane-catalyzed transesterification. The most often used acidic catalysts are Bronsted acids, especially sulfonic acids, sulfuric and hydrochloric acids. However, acid-catalyzed transesterification reactions require higher temperature and pressure of approximately 100 °C and 5 bar, respectively, and a longer reaction time (Maheshwari et al., 2022; Mumtaz et al., 2017).

Sodium, potassium hydroxides, as well as alkaline metals and alkoxides, are among the most frequently utilized alkaline catalysts in biodiesel production because of their affordability and user-friendliness, thus favouring industrial-scale biodiesel production.

Base-catalyzed transesterification reactions have a shorter reaction time than acid-catalyzed esterification reactions. However, alkaline catalysts are preferably for samples with low free fatty acids (FFAs) because of their high reactivity effects. Feedstocks with elevated FFAs can lead to soap formation when reacting with alkaline catalysts, disturbing the separation of glycerine, biodiesel and water phases (Maheshwari et al., 2022; Mumtaz et al., 2017).

Enzymatic-catalyzed transesterification reactions are attracted much in biodiesel production as the enzyme catalysts are natural, reusable, have a mild reaction condition requirement, and have specificity in multiple substrates. Lipases produced from microorganisms are the most employed enzymes as biocatalysts. For example, lipases extracted from yeast or fungi, such as *Aspergillus niger*, *Rhizopus oryzae* and *Candida antarctica* are commonly used in biodiesel synthesis (Kumar et al., 2023). The biocatalysts favour higher efficiency and better quality of biodiesel compared to acid and alkali catalysts, largely attributed to their superior characteristic, which is no soap formation occurred in the reaction. The drawbacks of enzymatic-catalyzed transesterification reactions are the high catalyst concentration required, low reaction rate, and high end-product recovery cost caused by the challenging enzyme separation process (Maheshwari et al., 2022; Mumtaz et al., 2017).

2.5.2 Factors influencing transesterification process

The transesterification process for biodiesel production is influenced by several factors, including water content, free fatty acids (FFAs), selection of alcohols, the ratio of alcohol to lipid, reaction temperature, reaction duration and catalyst concentration (Li et al., 2011). Water content and FFAs are crucial factors in this chemical process. The presence of large amounts of water and FFAs contents in raw lipids leads to a hydrolysis reaction of triglycerides, thus resulting in soap production, which diminishes the productivity and quality of FAME end products (Marchetti et al., 2007). The highest yield of biodiesel at 95% was achieved in 10-20% (v/v) water content (Thangaraj et al., 2018).

In alkali-catalyzed transesterification, the water content is unavoidable and always present in biodiesel feedstock as some water is produced during the reaction between base catalyst and alcohol. Thus, saponification reaction takes place, wherein water hydrolyses the triglycerides, yielding free fatty acids and glycerol. Then, the free fatty acid interacts with base catalyst, leading to soap formation. The reaction rate is decreased by the low catalyst concentration and catalytic effect due to consumption by saponification reaction. Meanwhile, gel and emulsion formation occurred, which caused the biodiesel to become more viscous. Thus, the product recovery downstream process becomes more challenging to separate the esters and glycerol due to soap formation. Therefore, the low levels of water and free fatty acids favour the transesterification reaction for biodiesel production (Li et al., 2011; Thangaraj et al., 2018).

Furthermore, the types of alcohols being utilized in transesterification, such as methanol, ethanol and butanol is also affecting the production of biofuels. Methanol is preferably used for biodiesel due to its superior characteristics over other alcohols. It provides a faster reaction and is easily separated from the end product. Besides, methanol has higher purity, lower cost and toxicity compared to other alcohols. On the other hand, ethanol is more costly, has a higher reaction time and lower product recovery. The ethanol reacts with water and forms an azeotropic mixture which requires an additional distillation process to separate esters from alcohol. Long-chain alcohols like 2-propanol and 1-butanol are also suggested for biodiesel synthesis. However, 2-propanol with branched chain requires a longer reaction time. Both long-chain alcohols require a relatively high temperature to dissolve the alkaline catalyst, thus lowering the biodiesel production efficiency (Maheshwari et al., 2022; Thangaraj et al., 2018).

Alcohol to lipid ratio determines the reversible transesterification reaction equilibrium. It has been established that the formation of 3 mol of alkyl ester and 1 mol of glycerol entails 3 mol of alcohol and 1 mol of triglyceride. According to the Le-Chateliers principle, the increases in reactant concentration will shift the equilibrium to oppose the changes, which increases the product concentration. Consequently, increasing the alcohol concentration will further increase the ester formation. Therefore, a higher molar ratio of alcohol to lipid will give a higher biodiesel yield. However, excess alcohol will react with acid catalyst, causing the recombination of glycerol and ester, which shifts the equilibrium backward and produces undesirable monoglycerides. If the alcohol to lipid ratio is beyond the optimum ratio, the separation process of glycerol and alcohol is complicated (Goh et al., 2022; Maheshwari et al., 2022).

Catalyst concentration affects the conversion of lipid to biodiesel. The increases in catalyst concentration increase the esters yield by accelerating the reaction rate. Transesterification reactions can be performed under mild conditions with the presence of catalysts. The reaction can also be conducted without catalyst under supercritical conditions such as a higher temperature requirement (Li et al., 2011). Hydroxide alkaline catalyst react with methanol to generate methoxide anions. Methoxide anions function as the active species and potent nucleophiles that target the carbonyl moiety in triglyceride compounds to generate alkyl ester (Salaheldeen et al., 2021). However, biodiesel conversion rate is decreased when the catalyst concentration is extremely high owing to the saponification reaction, which causes higher viscosity in reaction mixture, leading to difficult product recovery (Goh et al., 2022; Maheshwari et al., 2022).

The transesterification reaction is affected at different temperatures for different feedstocks. Increasing in temperature escalates the reaction rate and product yield increase. An elevated temperature enables the molecules have enough energy to overcome the activation energy barrier and increases the product yield. Temperature below 50 °C would produce a biodiesel with high viscosity. However, an extremely high temperature larger than 60 °C could evaporate the methanol, consequently reducing the product formation (Goh et al., 2022; Maheshwari et al., 2022). Besides, a higher biodiesel conversion rate is achieved with increased transesterification reaction time (Li et al., 2011; Maheshwari et al., 2022).

2.5.3 Biodiesel standards

G. lucidum lipid-derived biodiesel requires to adhere to the biodiesel standards, known as ASTM D6751-08 in the United States (American Society for Testing and Materials) and EN 14214 in Europe (European Norm), to be utilized in existing engines as a blend or substitute. Biodiesel standards are used to evaluate the characteristics of biodiesel samples. The composition and physio-chemical attributes of biodiesel depend on the used feedstock. Several authors determined that Ganoderma species exhibit higher levels of monounsaturated fatty acids (MUFA, C18:1) compared to polyunsaturated fatty acids (PUFA, C18:2) (Mohamad Jahis et al., 2022). Generally, fungi species with higher level of saturated fatty acid and monounsaturated fatty acid are favoured for biodiesel production (Rivaldi et al., 2017). Moreover, the biodiesel quality can be assessed by testing key parameters, for example, cetane number (CN), cold filter plugging point (CFPP), cloud point (CP), oxidation stability (OS), and iodine value (IV) (Knothe, 2017).

CP, PP and CFPP characterize the cold-flow characteristics exhibited by biodiesel samples (Nouri et al., 2019). The cloud point (CP) signifies the temperature when the first visible appearance of solid crystals occurs during the cooling process. This phenomenon is due to the crystallization of oil components in the biodiesel, such as waxes or other solid impurities, as the temperature decreases. Pour point (PP) is identified as the temperature where the fuel ceases to exhibit fluidity and does not flow readily (Knothe, 2017). Cold filter plugging point (CFPP) represents the temperature at which the fuel undergoes solidification, causing blockages in filters and fuel lines. CFPP value depends on the concentration of SFA, it tends to increase with high SFA and longer fatty chains. Biodiesel exhibiting higher CFPP values is more susceptible to filter and fuel line clogging at lower temperatures compared to biodiesel with lower CFPP values (Hussain et al., 2015; Rivaldi et al., 2017).

Iodine value (IV) is a crucial metric for indicating the degree of unsaturation within a biodiesel sample, reflects the total unsaturation, encompassing both MUFA and PUFA. The higher IV corresponds to an increased number of C=C double bonds (Knothe, 2017). IV is calculated based on the quantity of iodine in grams required to interact with double bonds present in 0.1 kg of samples. Hence, a higher IV indicates more available double bonds for oxygenation and the formation of hydroperoxides (Hussain et al., 2015). The hydroperoxides or other oxidized compounds can trigger polymerization of esters, leading to formation of insoluble deposits and gums, which contribute to fuel filter plugging (Rivaldi et al., 2017).

Cetane number (CN) serves as a key parameter to measure the combustion characteristics of biodiesel as a fuel in diesel engines, indicating both the ignition delay duration and ignition quality numerically. A higher CN is associated with a shorter ignition delay time, that is the fuel ignites more quickly during combustion process (Folayan et al., 2019; Hussain et al., 2015). CN exhibits significant variation depending on feedstock types, particularly in terms of fatty acid chain length and the composition of SFA, MUFA, and PUFA. Several authors have suggested the increased CN is directly proportional to SFA amount and the length of carbon chain. The existence of C=C bonds in the hydrocarbon chain shortens the carbon chain length, resulting in a lower CN and a longer ignition delay time. This prolongs the combustion process and reduces efficiency (Puhan et al., 2010; Tamilselvan et al., 2020).

Oxidation stability (OS) is a critical quality parameter to ensure the enduring storage and performance of biodiesel. Oxidation in biodiesel is a complex process involving three stages, which are initiation, propagation, and termination. Oxidation is initiated by the loss of hydrogen atoms, causing the creation of free radicals, which are extremely reactive entities that subsequently react with oxygen molecules in the atmosphere. This leads to the accumulation of peroxides, which are compounds containing an oxygen-oxygen single bond, followed by propagation and formation of hydroperoxides. Consequently, the breakdown of hydroperoxides into oxidation byproducts like aldehydes, and short-chain organic acids contributes to the deterioration of biodiesel quality (David et al., 2023; Masudi et al., 2023). Additionally, the induction period (IP) measures the stability of biodiesel, with a longer IP indicating a longer stability and higher resistance to oxidative degradation (Masudi et al., 2023).

The biodiesel oxidation stability is influenced by the storage conditions, including temperature, exposure to water, light, metal content and the presence of antioxidants. The composition of SFAs and total unsaturated fatty acids (UFAs), including both MUFA and PUFA also affects the oxidation stability. Several researchers suggested that biodiesel with higher UFAs and lower SFAs tends to exhibit lower oxidation stability, leading to poor atomization and combustion efficiency (David et al., 2023; Rivaldi et al., 2017).

2.6 Optimization study

In this study, the optimization of glucose concentration (g/L), agitation rate and pH level of *G. lucidum* will be accomplished to maximise the biomass and lipid production through the application of response surface methodology (RSM). RSM is a statistical technique widely employed for optimizing processes, giving various benefits in the context of experimental design and optimization. RSM needs a smaller number of experimental runs to attain the results compared to the conventional method, one-factor-at-a-time (OFAAT). In OFAAT optimization, each variable is examined independently while maintaining others constant, which requires separate experiments for each variable and results in a larger number of experiments (Elemary, 2019). In contrast, RSM allows researchers to efficiently explore the relationship between multiple variables and responses simultaneously. Additionally, RSM is designed to identify optimal conditions for a desired response. By systematically varying variables within an experimental region, researchers can determine the combination of variable levels that maximises or minimizes the responses (Veza et al., 2023). RSM employs statistical analysis of variance (ANOVA) to analyse the experimental results, which can evaluate the significance and identify the influential variables in the model (Nur Aishah et al., 2022). Also, RSM generates 3D response surface plots, visually representing the relationship between the variables and responses (Al-sharify et al., 2022).

Central composite design (CCD) and Box-Behnken design (BBD) are prevalent types of response surface design applied in RSM. CCD incorporates two-level or fractional factorial design points, including low, high, and center levels for each factor, along with center points to estimate experimental error. CCD involves axial points or star points located outside the factorial points to estimate curvature (Elemary, 2019). On the other hand, BBD consists of three levels for each factor, including center points, and is particularly useful for fitting a second-degree polynomial equation to the experimental data (Veza et al., 2023).

Overall, the fermentation conditions are required to be investigated for obtaining the maximum biomass and lipid yield in both shake-flake and bioreactor fermentation. It is now well-established that *G. lucidum* mycelial biomass could be the potential feedstock for biodiesel. Biodiesel derived from *G. lucidum* mycelial biomass has the advantages of being less time-consuming and having no land requirement. This indicates a need for a closer look at the production and optimization of *G. lucidum* mycelial biomass lipid in a specialized bioreactor.

CHAPTER 3 METHODOLOGY

The overview of the methodology conducted in this study was depicted in Figure 3.1. The *G. lucidum* mycelium strain QRS 5120 was selected and subcultured on PDA plates. The mycelium was then cultivated in standardized media through submerged-liquid fermentation. To optimize fermentation conditions, response surface methodology (RSM) with central composite design (CCD) was employed, evaluating on variables such as initial pH, glucose concentration, and agitation rate. The optimized conditions were verified in both 500 mL shake flasks and a 3 L Air-L-Shaped Bioreactor (ALSB). The methodology also included analysis of pellet morphology, lipid extraction through solvent extraction, and gas chromatography for fatty acid composition. Finally, the extracted lipids were converted to biodiesel via acid-catalyzed transesterification, and the biodiesel properties were assessed to ensure compliance with international standards.

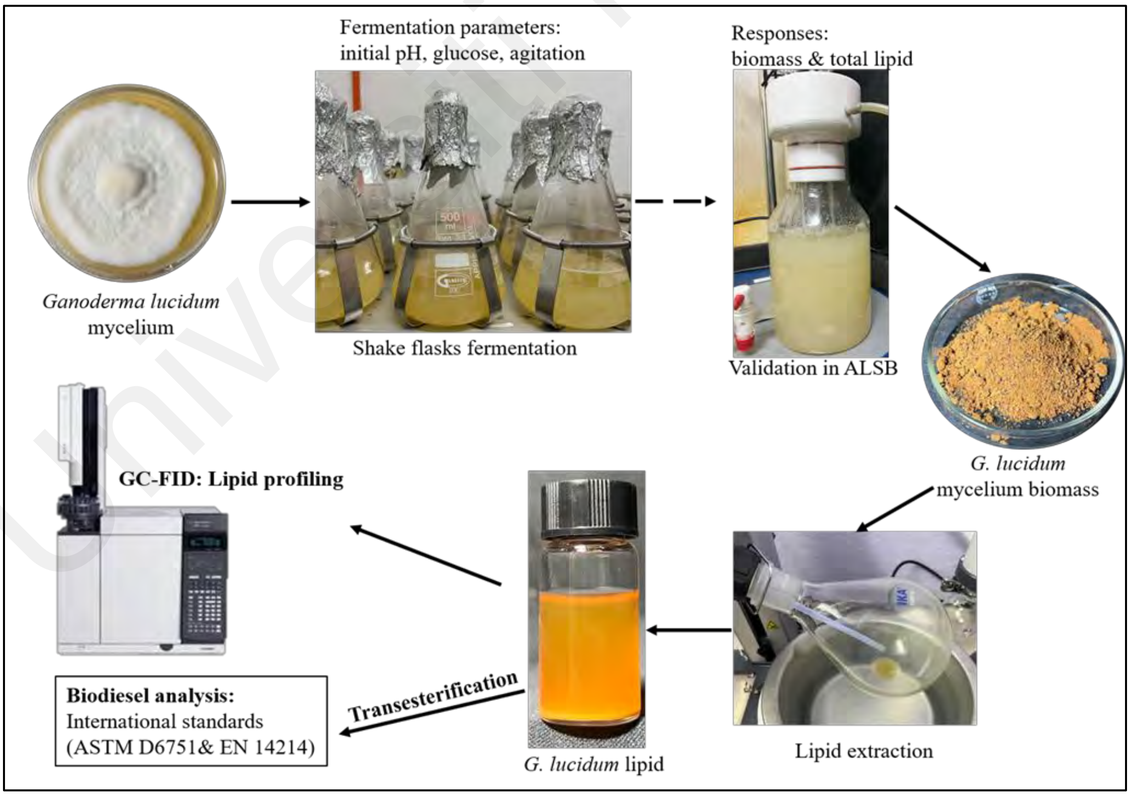

Figure 3.1: An overall methodology flow.

3.1 Strain selection and media composition

G. lucidum strain QRS 5120 was provided by Functional Omics and Bioprocess Development Laboratory, Institute of Biological Sciences, University Malaya. The *G. lucidum* mycelium was cut from the mother plate with the dimension of 1 cm x 1 cm, followed by subculturing on the potato dextrose agar (PDA) plate. Incubation of the culture plates took place at ambient temperature for 7 days. The culture plates were kept for further use in batch fermentation. The medium composition for two seed cultures used in all stages of fermentation is constant at (g/L): Yeast Extract (1), NH_4Cl (4), $MgSO_4$ (0.5), KH_2PO_4 (0.5) and K_2HPO_4 (0.5), unless explicitly stated otherwise (Abdullah et al., 2020).

3.2 Submerged-liquid fermentation

The submerged-liquid fermentation was carried out as demonstrated by Hassan et al. (2019) with minor modifications. The inoculum was prepared in a 500-mL shake flask accordingly to the media composition and fermentation conditions in Table 3.1. The parameters for the media were standardized as follows: agitation ranging from 50-150 rpm, glucose concentration between 10 g/L and 50 g/l, and initial pH 4-6. The first seed culture was prepared by cutting 3 mycelial agar squares and introducing them into a 500 mL shake flask containing 100 mL medium. The first seed culture was incubated for 10 days. Then, the first seed culture containing mycelium pellet was homogenised using a sterile Waring hand mixer. After that, 40 ml (20% v/v) of the blended mycelium was introduced into a new 500 ml shake flask containing 160 mL fresh medium (second seed culture) and incubated for another 10 days.

3.3 Optimization of fermentation conditions using RSM

The fermentation conditions of *G. lucidum* including initial pH, glucose (g/L) and agitation (rpm) were optimized using RSM. RSM with CCD was selected to establish experimental sets in Design Expert version 13.0 software. The experimental range and levels of the variables for this research is shown in Table 3.1. The alpha value was set to 1.0. The lowest level for variables were initial pH, pH 4; glucose concentration, 10 g/L; and agitation rate, 50 rpm; and the highest level were initial pH 6; glucose concentration, 50 g/L; and agitation rate, 150 rpm. Then, 20 experiments were formulated utilizing CCD design with selected variables and responses, as indicated in Table 3.2. All experiments were performed in triplicate.

Table 3.1: Experimental range and levels of the independent variables.

Independent variables	Range and levels		
	-1	0	1
Initial pH	4	5	6
Glucose (g/L)	10	30	50
Agitation (rpm)	50	100	150

To investigate the influence of variables and their reciprocation, an empirical model was developed using a second-order quadratic model for the responses as demonstrated in Equation 1:

$$Y = b_0' + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j>1}^{n} b_{ij} X_i X_j \qquad (1)$$

where Y is the predicted response, b_0' is the constant coefficient, b_i is the linear coefficient, b_{ij} is the interaction coefficient, b_{ii} is the quadratic coefficient, and $X_i X_j$ are the coded values.

Table 3.2: Experiments generated from RSM with CCD design according to selected independent variables and responses.

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	A: Initial pH	B: Glucose g/L	C: Agitation rpm	Biomass g/L	Total lipid g/L
1	4	30	100		
2	4	50	150		
3	6	10	50		
4	4	50	50		
5	5	30	100		
6	5	10	100		
7	5	30	100		
8	5	30	150		
9	4	10	150		
10	5	50	100		
11	5	30	100		
12	5	30	100		
13	6	10	150		
14	5	30	50		
15	6	50	150		
16	5	30	100		
17	6	50	50		
18	5	30	100		
19	4	10	50		
20	6	30	100		

3.4 Verification of optimized model in 500-ml shake flask and 3 L Air-L-Shaped Bioreactor (ALSB)

The RSM was applied to develop the optimized models for maximising biomass and lipid production of *G. lucidum*. The optimized models were verified in 500-mL shake flask fermentation in triplicate. The verification experiment for biomass and lipid production was carried out in a 3 L ALSB for the consideration of the feasibility to scale-up the *G. lucidum* cultivation for biodiesel production. The 20% inoculum from the second seed culture was transferred into the ALSB with 2 L of total working volume (w/v). The verification experiments for shake flasks and bioreactors were performed in the dark for 10 days. The cultured mycelium was harvested on day 10 for the calculation of total biomass and further lipid production (Supramani et al., 2023).

3.5 Pellet morphology

An optical microscope (CX23LEDFRS1, Hamburg, Germany) was employed to analyse the morphology of sample pellets. A 10 mL culture sample was collected and placed into a petri dish. The pellets from each optimized model were chosen randomly from the petri dish and positioned onto a glass slide for microscope observation at 4 x magnification. The pellet morphology from each culture was observed in triplicate (Usuldin et al., 2023).

3.6 Lipid extraction

Lipid extraction for *G. lucidum* mycelial biomass was performed through solvent extraction method according to Mohamad Jahis et al. (2022) with minor modifications. The mycelium biomass was dried at 60 °C for 3 days in a food dehydrator. Next, the dried mycelium biomass was ground into a fine powder. The lipid was extracted using hexane at a 1:100 (g/mL) of biomass to solvent ratio. The biomass powder was soaked in a 1 L beaker containing hexane and placed on a hot plate. Then, the sample was stirred for 2 hours using a magnetic stirrer at 60 °C. The sample was filtered using Whatman GF/C glass fibre filter, and the excess hexane was evaporated using a rotary evaporator to obtain the lipid.

3.7 Gas chromatography analysis

The fatty acid composition of *G. lucidum* lipid was determined in accordance with AOAC 996.06 (AOAC, 2016). The analysis was conducted on an Agilent 7890A gas chromatograph (GC) (Agilent Technologies, Palo Alto, CA) equipped with a flame-ionization detector (FID). DB-225 capillary column (30 m x 250 μm x 0.25 μm) (Agilent Technologies) was used. The findings were indicated as a percentage relative to the total peak area encompassing all fatty acids present in the *G. lucidum* lipid extract. The fatty acids were determined by comparing the retention times in the sample with the FAME standard.

3.8 Analytical methods

3.8.1 Determination of mycelium biomass

The pre-dried and pre-weighed filter paper was used to filter the fermentation broth. The fermentation broth was filtered through a Buchner funnel filter set and the mycelial biomass was washed repeatedly with distilled water. Subsequently, the mycelial biomass was dried in a food dehydrator at 60 °C until reaching a consistent weight. The dry weight of mycelial biomass was obtained after deducting the pre-weighed filter paper (Abdullah et al., 2020).

3.8.2 Lipid yield determination

The extracted lipid will remain dissolved in the hexane solvent, which was subjected to rotary evaporation to remove excess hexane. The mass of lipid remaining was weighed after the evaporation of hexane. The extracted lipid yield was calculated by applying the formula as Eq 2 (Mohamad Jahis et al., 2022).

$$\text{Lipid yield (\%)} = \frac{m_i}{m_s} \times 100 \tag{2}$$

whereas the coefficient mi (g/L) is the weight of recovered lipid while ms (g/L) is the weight of pre-dried and pre-weighed biomass used for SVE extraction.

3.9 Statistical analysis

The internal statistical tool in Design Expert version 13.0 software (Stat-Ease, Minneapolis, USA) was applied to conduct analysis of variance (ANOVA) for the CCD quadratic model. The statistical significance for each of the model coefficients will be presented by p-values < 0.05.

3.10 Biodiesel production through transesterification

Acid-catalyzed transesterification as outlined by Redzwan et al. (2017) with some modifications was utilized to convert the extracted *G. lucidum* lipid to biodiesel. The transesterification process was executed in a laboratory scale by using a 1 L round-bottomed glass flask. Sulphuric acid was selected as the acidic catalyst while anhydrous methanol (99%) was employed as the reactant. The reaction product was evaporated by rotary evaporator at 70 °C for 20 minutes. Then, gravity settling using separating funnel was performed to separate the upper layer fatty acid methyl ester (FAME) from glycerol at lower layer. The FAME was washed with distilled water for catalyst removal, followed by a purification step with silica gel, and dehydration using anhydrous sodium sulphate.

3.11 Determination of biodiesel properties

To determine the compliance of obtained *G. lucidum* biodiesel (Ganodiesel) with the standard specifications, the biodiesel characteristics of Ganodiesel were examined by conducting various tests following the international guidelines (CEN, 2019; ASTM D6751-08, 2018). The following tests, including cloud point, kinematic viscosity, ignition point, oxygen stability and density were conducted. Kinematic viscosity was measured on Automatic Kinematic Viscosity Measuring System AKV-201 at 40 °C in accordance to ASTM D445. The cloud point (ASTM D6749) was determined using Mini Cloud Point Tester MPC-102 in a temperature of -60 °C to 51 °C. For the ignition point, the test was conducted using Pensky-martens Closed Cup Automated Flash Point Tester APM-7 in accordance to ASTM D93. All these tests were conducted using certified instruments provided by TANAKA Scientific Ltd., Tokyo. Oxidation stability was tested on Rancimat 743 (Methrom, Herisau, Switzerland) in accordance to EN 14112. Besides, tests for monoglyceride, triglyceride, diglyceride, ester content, total glycerol content and acid number were measured under European Standard Methods (CEN, 2019).

CHAPTER 4 RESULTS AND DISCUSSION

Chapter 4 presented a comprehensive analysis of the optimization of biomass and lipid production from *G. lucidum* using RSM with CCD. The chapter detailed the experimental sets, including the influence of initial pH, glucose concentration, and agitation rate on biomass and lipid yields. Optimization experiments, designed through CCD, resulted in predictive models for both biomass and lipid production, with statistical validation using analysis of variance (ANOVA). The significant effects of variables on biomass and lipid yields were illustrated through 3D response surface plots. The chapter also discussed the verification of optimized conditions in both shake flasks and ALSB. Additionally, the fatty acid profile of the *G. lucidum* lipid was compared to commercial feedstocks, and the biodiesel properties were evaluated against international standards. Finally, the chapter concluded with observations on pellet morphology under different fermentation conditions and a comparison of this study's findings with existing literature.

4.1 Optimization

By employing RSM, experiments were conducted to assess the effect of initial pH, glucose concentration (g/L) and agitation rate on biomass and lipid production. CCD was employed with the levels of each variable and responses detailed in Table 4.1. Twenty experiments were developed by CCD, where the coefficients were analysed using non-linear regression analysis. The significance of the model coefficient was assessed using analysis of variance (ANOVA). Statistical significance of individual coefficients was determined by a *p*-value of less than 0.05.

Table 4.1: Experimental design matrix using RSM with CCD and responses for the mycelial biomass (DCW) and lipid production from *G. lucidum*.

Run No.	Variables			Responses			
	Initial pH (A)	Glucose (g/L) (B)	Agitation (rpm) (C)	Biomass (DCW g/L)		Total Lipid (%)	
				Actual	Predicted	Actual	Predicted
1	4	30	100	5.28	6.03	2.90	2.77
2	4	50	150	2.30	2.46	2.19	2.26
3	6	10	50	1.03	0.81	4.00	3.98
4	4	50	50	3.95	3.64	2.94	3.02
5	5	30	100	6.07	6.40	2.92	3.11
6	5	10	100	3.67	4.25	3.96	4.01
7	5	30	100	6.87	6.40	3.33	3.11
8	5	30	150	3.54	3.53	2.48	2.41
9	4	10	150	1.93	1.46	2.33	2.30
10	5	50	100	7.07	6.74	3.68	3.39
11	5	30	100	6.81	6.40	2.96	3.11
12	5	30	100	6.26	6.40	3.38	3.11
13	6	10	150	2.07	2.31	3.98	3.96
14	5	30	50	3.10	3.37	2.98	2.81
15	6	50	150	4.70	4.78	3.54	3.58
16	5	30	100	6.77	6.40	2.93	3.11
17	6	50	50	4.40	4.80	2.69	2.78
18	5	30	100	6.12	6.40	2.64	3.11
19	4	10	50	1.27	1.13	3.88	3.89
20	6	30	100	7.53	7.03	3.58	3.48

4.2 Optimization of mycelium biomass production

The ANOVA analysis for mycelium biomass production is presented in Table 4.2. The predicted coefficient determination reveals that 96.83% ($R^2 = 0.9683$) of the variability in the actual response can be explained using this model. The model is significant ($p < 0.005$). The adjusted coefficient determination (Adj. $R^2 = 0.9397$) further supports the model's significance and is reasonably consistent with the predicted R^2 value (0.7553), differing by less than 0.2. The model for biomass yield was derived by considering the actual variables and is expressed in Eq (3). For example, the positive interaction coefficient (+0.0184906) between initial pH and glucose indicates that the interaction of these variables positively affects biomass production. The negative linear coefficient (-1.945160) for initial pH suggests that an increase in initial pH leads to a decrease in biomass production.

$$\textbf{\textit{Biomass}} = -4.16075 - 1.94516 \times \textit{Initial pH} + 0.143333 \times \textit{Glucose} + 0.220156 \times \textit{Agitation} + 0.0184906 \times \textit{Initial pH} \times \textit{Glucose} + 0.00582375 \times \textit{Initial pH} \times \textit{Agitation} - 0.000379712 \times \textit{Glucose} \times \textit{Agitation} + 0.130877 \times \textit{Initial pH}^2 - 0.00225856 \times \textit{Glucose}^2 - 0.00118147 \times \textit{Agitation}^2 \quad (3)$$

Table 4.2: Analysis of variance (ANOVA) for the experimental results of the CCD quadratic model for *G. lucidum* mycelium biomass.

Source	Sum of Squares	df	Mean Square	F-value	Prob > *F*	
Model	81.79	9	9.09	33.9	< 0.0001*	significant
A-Initial pH	2.51	1	2.51	9.35	0.0121*	significant
B-Glucose	15.53	1	15.53	57.92	< 0.0001*	significant
C-Agitation	0.0632	1	0.0632	0.2356	0.6378	
AB	1.09	1	1.09	4.08	0.0710	
AC	0.6783	1	0.6783	2.53	0.1428	
BC	1.15	1	1.15	4.3	0.0648	
A^2	0.0471	1	0.0471	0.1757	0.6839	
B^2	2.24	1	2.24	8.37	0.016*	significant
C^2	23.99	1	23.99	89.5	< 0.0001*	significant
Residual	2.68	10	0.2681			
Lack of Fit	1.99	5	0.3981	2.88	0.135	not significant
Pure Error	0.6904	5	0.1381			
Cor Total	84.47	19				
Std. Dev. = 0.5178		R^2 = 0.9683			Adeq Precision = 16.9985	
Mean = 4.54		Adjusted R^2 = 0.9397				

* Significant value.

From the model, glucose (B) demonstrates the strongest effect ($p < 0.0001$) on biomass, whereas initial pH (A) shows a significant effect at $p < 0.05$. The quadratic terms of glucose (B^2) and agitation (C^2) indicate a significant effect at $p < 0.05$ on the production of mycelium biomass. Conversely, agitation (C) and the quadratic terms (A^2, AB, AC and BC) exhibit negative effects. Figure 4.1 displays the combined effect of initial pH, glucose concentration and agitation in three-dimensional (3D) surface. Figure 4.1a depicts the effect of initial pH (A) and starting glucose concentration (B), Figure 4.1b depicts the effect of A and agitation rate (C), and Figure 4.1c depicts the effect of B and C on biomass production. Figure 4a indicates that an increase in both pH and glucose levels corresponds to a simultaneous increase in biomass production, suggesting a collaborative effect between pH and glucose. Figure 4.1b demonstrates that maintaining agitation at an intermediate level, along with an increase in pH, causes a higher production of biomass. Meanwhile, in Figure 4.1c, it is shown that the moderate agitation speed, coupled with an increase in glucose concentration, results in a higher production of biomass. The maximum biomass yield (8.66 g/L) was obtained at initial pH 5.88, 44.88 g/L glucose concentration and 97 rpm.

The initial pH of the medium affects the availability and solubility of nutrients required for mycelial growth, thus influencing the consumption rates during fermentation (Arana-Gabriel et al., 2020). Glucose acts as the primary carbon source for mycelial propagation and development. Therefore, the availability and concentration of glucose in fermentation medium directly impact the mycelium biomass production (Pessoa et al., 2023). Low agitation speed limits the dissolved oxygen levels in the medium, hindering mycelium oxygenation and consequently affecting mycelium growth. Conversely, excessively high rotational speeds can induce mechanical stress on the mycelial structures, leading to mycelial autolysis and reducing biomass productivity (Cui & He, 2012).

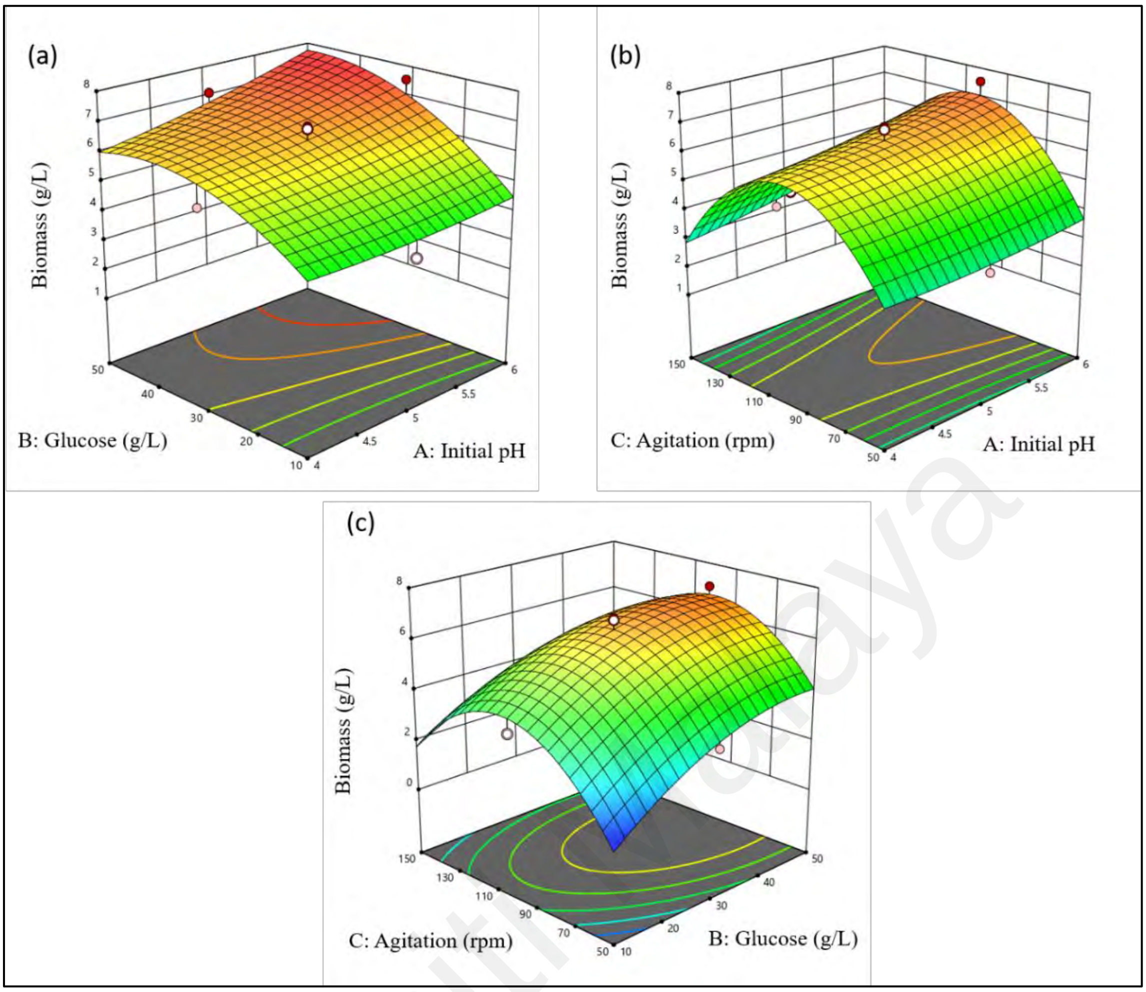

Figure 4.1: 3D response surface plot of mycelium biomass from *G. lucidum* indicating the interaction between (a) Initial pH and glucose, (b) Initial pH and agitation, (c) Glucose and agitation.

4.3 Optimization of lipid production

The ANOVA results for lipid production of *G. lucidum* is displayed in Table 4.3. The predicted coefficient determination reveals that 90.01% ($R^2 = 0.9001$) of the variability in the actual response can be elucidated using this model. The *p*-value of 0.0020 ($p < 0.005$) signifies that the model is statistically significant. The adjusted coefficient determination (Adj. $R^2 = 0.8102$) further supports the model's significance and closely aligns with the predicted R^2 value (0.7226), differing by less than 0.2. The regression model based on the actual factor of lipid can be expressed using Eq (4).

$$\boldsymbol{Lipid} = 5.87637 - 0.474875 \times Initial\ pH - 0.104437 \times Glucose - 0.0095075 \times Agitation - 0.0041875 \times Initial\ pH \times Glucose + 0.0078 \times Initial\ pH \times Agitation + 0.00020625 \times Glucose \times Agitation + 0.0175 \times Initial\ pH^2 + 0.0014875 \times Glucose^2 - 0.000198 \times Agitation^2 \quad (4)$$

Table 4.3: Analysis of variance (ANOVA) for the experimental results of the CCD quadratic model for total lipid production from *G. lucidum* mycelium.

Source	Sum of Squares	df	Mean Square	F-value	Prob > F	
Model	5.46	9	0.6061	10.01	0.0006*	significant
A-Initial pH	1.26	1	1.26	20.76	0.0010*	significant
B-Glucose	0.961	1	0.961	15.88	0.0026*	significant
C-Agitation	0.3842	1	0.3842	6.35	0.0304*	significant
AB	0.0561	1	0.0561	0.9271	0.3583	
AC	1.22	1	1.22	20.1	0.0012*	significant
BC	0.3403	1	0.3403	5.62	0.0392*	significant
A^2	0.0008	1	0.0008	0.0139	0.9084	
B^2	0.9736	1	0.9736	16.08	0.0025*	significant
C^2	0.6738	1	0.6738	11.13	0.0075*	significant
Residual	0.6053	10	0.0605			
Lack of Fit	0.2114	5	0.0423	0.5368	0.7444	not significant
Pure Error	0.3938	5	0.0788			
Cor Total	6.06	19				
Std. Dev. = 0.246		R^2 = 0.9001			Adeq Precision = 10.048	
Mean = 3.16		Adjusted R^2 = 0.8102				

* Significant value.

The model indicates that both initial pH (A) and glucose concentration (B) exhibited a remarkably significant effect at $p < 0.005$. Agitation (C) shows a significant effect at $p < 0.05$. Among the three variables, initial pH showed the highest significant ($p = 0.0010$), followed by glucose concentration ($p = 0.0026$) and agitation rate ($p = 0.0304$). The quadratic terms (AC, BC, B^2 and C^2) also showed a significant effect ($p < 0.05$) on total lipid production. Conversely, quadratic terms (AB and A^2) show negative effect. Figure 4.2 illustrates the combined effect of initial pH, glucose concentration and agitation in response surface curve. Figure 4.2a shows the effect of initial pH (A) and glucose concentration (B), Figure 4.2b shows the effect of A and agitation (C), and Figure 4.2c shows the effect of B and C on total lipid production. Figure 4.2a and c show that a reduction in glucose concentration results in an increase in total lipid production. By increasing the initial pH, as depicted in Figure 4.2a and c, enhanced the total lipid yield.

According to Figures 4.2b and 4.2c, the agitation rate significantly impacts the total lipid yield. Both figures demonstrate that total lipid production decreases at both lower and higher agitation rates, with the most favourable lipid yield observed within 90 to 110 rpm. The maximum total lipid yield (3.62%) was obtained at initial pH 4.68, 10.11 g/L glucose concentration and 68 rpm.

The speed of agitation could influence the condition of mycelium cells, leading to both beneficial and unfavourable effects. Increased agitation positively impacts oxygen levels and nutrient solubility, ensuring uniform distribution, which in turn fosters increased lipid production. In contrast, an excessively high agitation rate can have a detrimental impact by inducing shear stress and damaging the mycelium cells (Miranti et al., 2018). For example, Adriana Tita & Miftahul (2023) suggested that maintaining the agitation rate at 100 rpm, ranging from 0 to 200 rpm, resulted in an optimal increase in lipid yield of fungal BR 2.2 isolate. Moreover, a higher lipid yield observed at a lower glucose concentration might be due to the nutrient limitation or starvation, which caused an environment stress that prompts cells to accumulate lipid as a response. The impact of initial pH on lipid production could be because of its influence on cell growth and nutrient uptake (Robles-Iglesias et al., 2023).

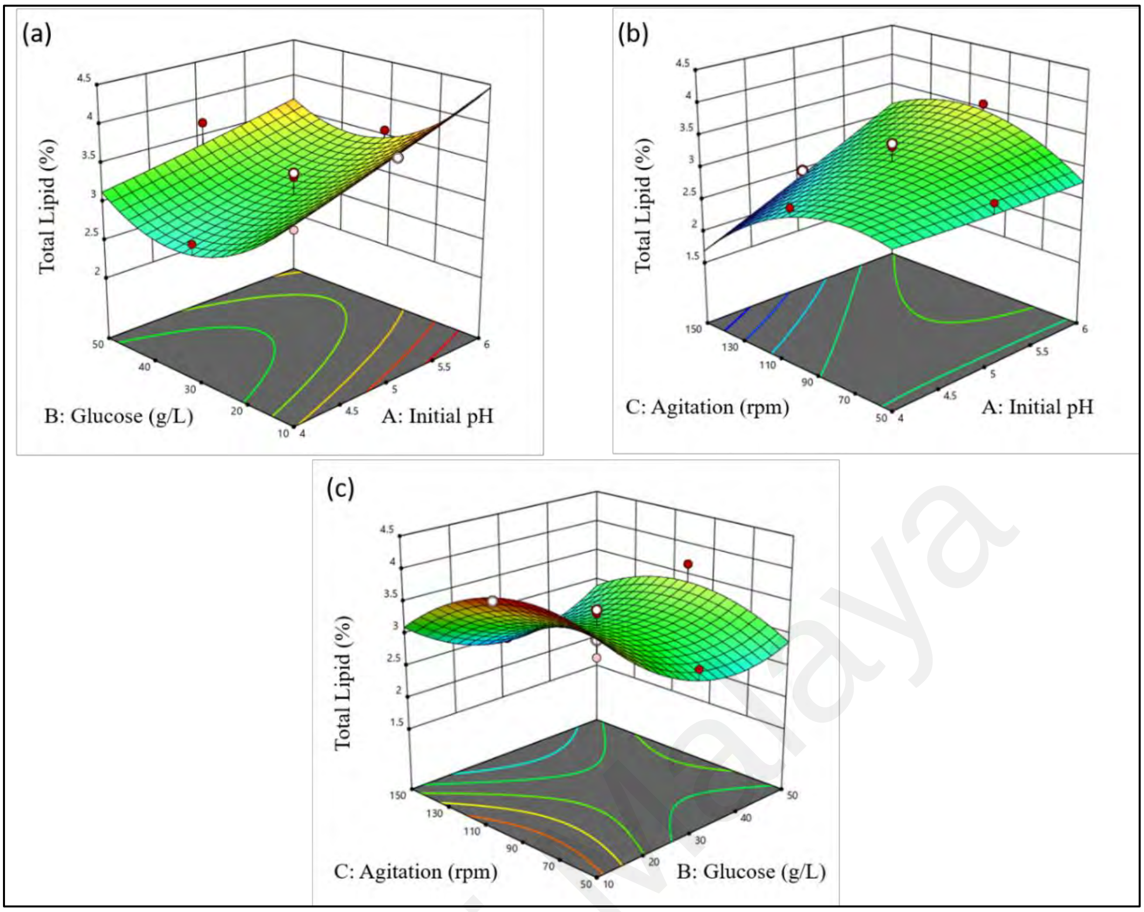

Figure 4.2: 3D response surface plot of total lipid production from *G. lucidum* mycelium biomass indicating the interaction between (a) Initial pH and glucose, (b) Initial pH and agitation, (c) Glucose and agitation.

4.4 Verification of the optimized conditions

After the completion of developing the CCD quadratic model for biomass and total lipid production, the optimization of both responses for *G. lucidum* was conducted using Design Expert 13.0 software. Table 4.4 shows the optimized conditions applied to verify the mycelium biomass and total lipid production in statistical models. To validate the robustness and accuracy of the model as per Eq 3 and Eq 4, the validation experiments were conducted in 500-ml shake flasks and 3 L Air-L-Shaped Bioreactor (ALSB) under controlled conditions. Maximizing both biomass and total lipid production was achieved using the parameters of initial pH 6, 50 g/L glucose concentration and agitation at 113 rpm. This resulted in 8.33 g/L of biomass and 2.17% of total lipid in shake flasks, whereas ALSB produced 5.32 g/L of biomass and 2.35% of total lipid. The total lipid production in ALSB was 1.1-fold increase compared to that in the shake flaks. Nevertheless, the biomass yield in ALSB was lower compared to shake flasks. The cultivation factors, including dissolved oxygen level, as well as agitation rate affected the productivity of both biomass and lipid in ALSB. The use of L-shaped impeller and supplied of oxygen in ALSB provided aeration and potentially resulting in increased lipid production by the mycelium (Sohedein et al., 2020; Usuldin et al., 2023). The study conducted by Saad et al. (2014) on the lipid production of *Cunninghamella bainieri* 2A1 reported that aeration facilitates the nutrient mixing and supplies dissolved oxygen in the fermentation medium, resulting in increased lipid production. Aeration also positively impacts the consumption of sugar by lipid-producing fungi. However, the biomass produced in ALSB in this study was lower, likely due to suboptimal parameters for biomass production (Supramani et al., 2023). Additionally, Figure 4.3 depicts the upstream and downstream processes of *G. lucidum* submerged liquid fermentation in 500 mL-shake flasks and 3 L ALSB for biomass and lipid production.

Table 4.4: Validation of the model with the optimized conditions.

To maximise	Variables			Predicted Responses		Actual Responses	
	Initial pH	Glucose (g/L)	Agitation (rpm)	Biomass (DCW g/L)	Total Lipid (%)	Biomass (DCW g/L)	Total Lipid (%)
Biomass	5.88	44.88	97	7.60	-	8.66 (SF)*	-
Total lipid	4.68	10.11	68	-	4.00	-	3.62 (SF)
Biomass and Total lipid	6	50	113	7.53	3.75	8.33 (SF) 5.32 (ALSB)*	2.17 (SF) 2.35 (ALSB)

*SF = 500 mL shake flask, ALSB = 3 L Air-L-Shaped Bioreactor.

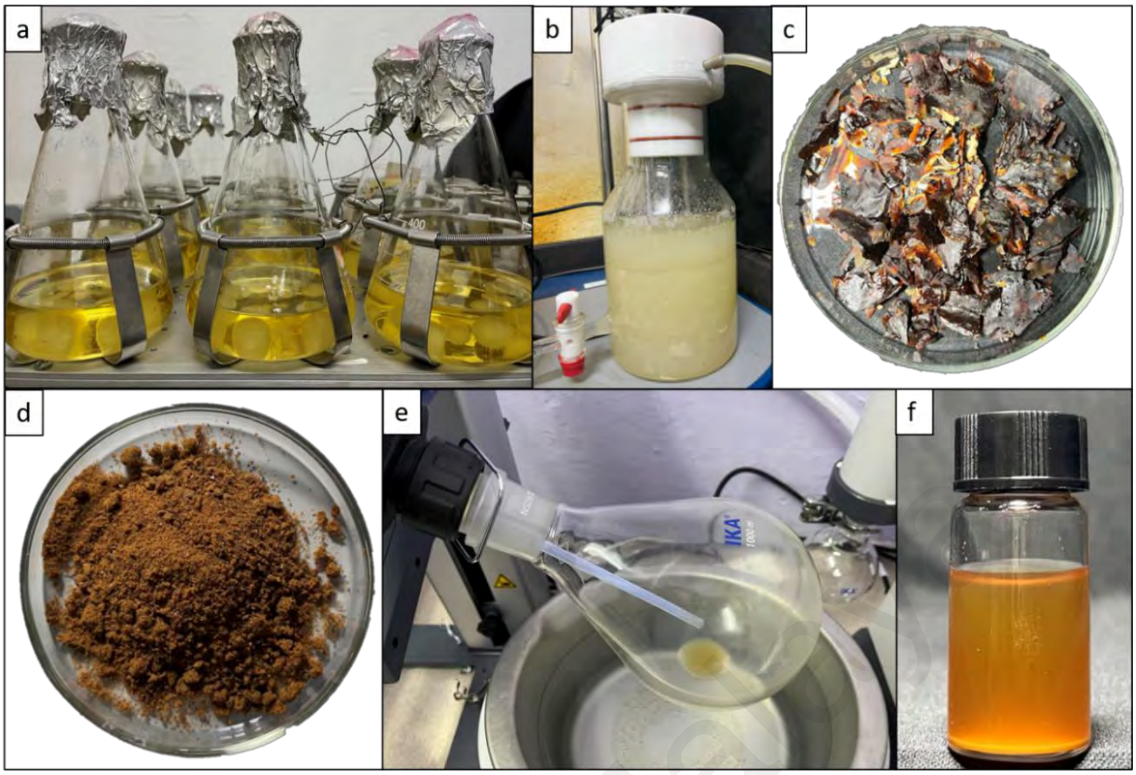

Figure 4.3: Overview of processes in *G. lucidum* SLF for biomass and lipid production: (a) first seed culture fermentation in 500 mL shake flasks, (b) second seed culture fermentation in a 3 L ALSB, (c) dried mycelium biomass, (d) powdered mycelium biomass, (e) *G. lucidum* lipid collected after removing excessive solvent using rotary evaporator, and (f) *G. lucidum* lipid.

4.5 *Ganoderma lucidum* fatty acid profiles

Gas chromatography result for *G. lucidum* lipid is indicated in Table 4.5. The FAME profiles of *G. lucidum* lipid from this study were compared with several basidiomycetes and commercial biodiesel feedstocks. The analysis revealed that fatty acids with chain lengths from C16 to C18 were the most consistently detected, as shown in the chromatogram result in Figure 4.4. The dominant fatty acids in *G. lucidum* lipid were palmitic acid (C16:0) at 18.60%, oleic acid (C18:1) at 16.21%, and linoleic acid (C18:2) at 48.11%. This fatty acid composition aligns with *G. lucidum* lipid profiles reported by Tel-Cayan et al. (2017). Sharipova et al. (2016) also found that fatty acids in several basidial fungi species, which studied for biodiesel purposes were predominantly comprised of palmitic, oleic, and linoleic acids. Generally, *G. lucidum* FAME profiles are characterized by the prevalence of C16 and C18 carbon series, which are crucial fatty acids in the context of biodiesel production. The key fatty acids that determine biodiesel quality included stearic acid, oleic acid, palmitic acid, linoleic acid and linolenic acid (Hawrot-Paw et al., 2021). Fatty acids that are saturated exhibit high resistance to degradation, thereby contributing to the durability of biodiesel. Additionally, saturated fatty acids play a role in enhancing resistance of biodiesel to oxidation in high-temperature environments (Talebi et al., 2013). On the other hand, unsaturated fatty acids (UFAs) such as C18:1 and C18:2 can improve cold flow properties. Several studies suggested that an increased concentration of unsaturated fatty acids tends to perform better in cold weather. It maintains fluidity at lower temperature, ensuring reliable engine performance and fuel system operation. (Sierra-Cantor et al., 2017; Verma et al., 2016). In this study, the fatty acid methyl ester profiles of *G. lucidum* lipid are closely resembled that of vegetable oils used in biodiesel production, which are soybean and cottonseed oils. This similarity implies the potential of *G. lucidum* lipid as the source of biodiesel production, providing a comparable quality to commercial oil feedstocks.

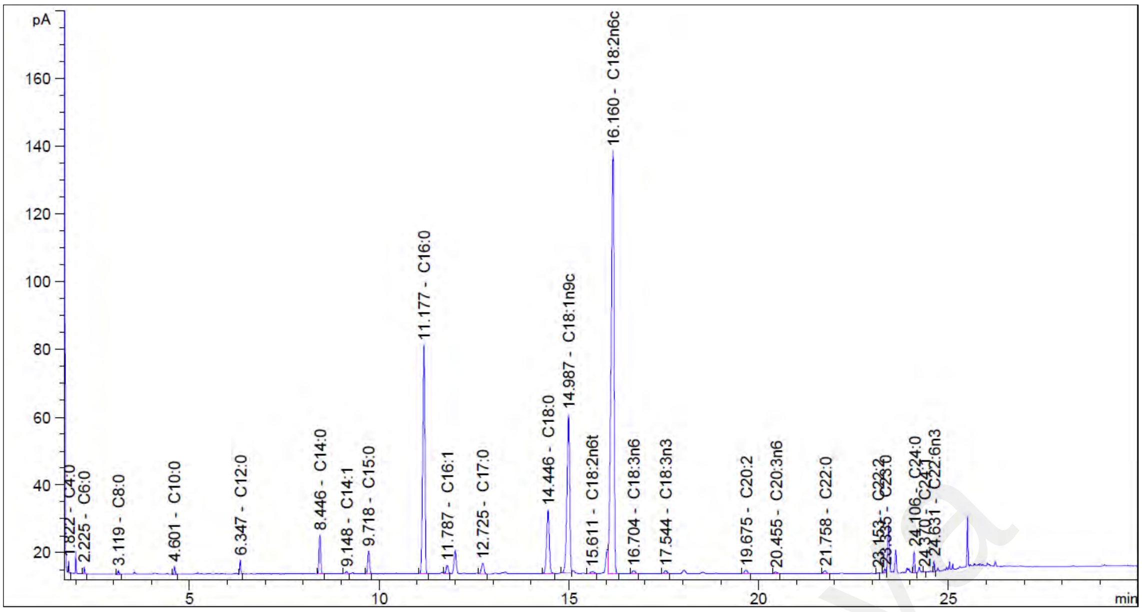

Figure 4.4: GC-FID chromatogram of fatty acid composition from *G. lucidum* lipid.

Table 4.5: Comparison of fatty acid methyl ester composition of *G. lucidum* lipid with other feedstocks.

Strains	Fatty acids (%)							References
	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	α-Linolenic acid (C18:3)	Arachidic acid (C20:0)	
Ganoderma lucidum	18.60	0.70	6.44	16.21	48.11	0.34	0.00	This study
Piptoporus betulines	11.50	0.67	2.99	36.60	47.80	-	-	(Sharipova et al., 2016)
Boletus appendiculatus	19.50	-	10.40	15.00	54.20	-	0.14	
Trametes versicolor	10.40	-	2.68	24.70	61.20	-	-	
Commercial oils								
Soybean oil	10-12	-	3-5	18-26	49-57	6-9	-	(Srinivasan et al., 2021)
Rapeseed oil	2-6	-	4-6	52-65	18-25	10-11	-	
Jatropha	14.62	1.47	7.36	41.43	35.42	0.20	0.30	(Sajjadi et al., 2016)
Cotton seed	26.23	-	1.30	13.30	59.13	-	-	

4.6 Biodiesel properties of *Ganoderma lucidum* FAME

Table 4.6 compares the physical characteristics of *G. lucidum* FAME (Ganodiesel) with international biodiesel standards. The findings indicate that Ganodiesel aligns with the biodiesel properties outlined by the US (ASTM D6751-08) and EU (EN 14214) standards. The parameters, including ignition point (172 °C), cloud point (-1.5 °C), kinematic viscosity (4.5 mm^2/s), oxidation stability (8 h), acid value (0.35 mg KOH/g), FAME content (97.2%), and total glycerol content (0.18%) have been determined to meet the specified range established by international standards. However, the density of 835 kg/m^3 does not meet the standards, which might be due to the higher proportion of unsaturated fatty acids (Talebi et al., 2013).

Table 4.6: Comparison of *G. lucidum* FAME biodiesel properties with international standards [a].

Properties	Unit	Method	FAME (Ganodiesel)	EU (EN 14214)	US (ASTM D6751-08)
Kinematic viscosity (40°C)	mm^2/s	ASTM D445	4.5	3.5-5.0	1.9-6.0
Cloud point	°C	ASTM D6749	-1.5	-	-3.0 to 12.0
Ignition point	°C	ASTM D93	172	≥120	≥130
Density	kg/cm^3	ASTM D4052	835	860-900	-
Oxidation stability	h	EN 14112	8	≥6	≥3
Acid value	mg KOH/g	EN 14104	0.35	<0.8	<0.5
FAME Content	wt%	EN 14103	97.2	*Min 96.5	-
Total Glycerol	wt%	EN 14105	0.18	*Max 0.25	Max 0.24
Monoacylglycerol	wt%	EN 14105	0.45	Max 0.8	-
Diacylglycerol	wt%	EN 14105	0.18	Max 0.2	-
Triacylglycerol	wt%	EN 14105	0.12	Max 0.2	-

[a] CEN (2019); ASTM D6751-08 (2008)
* Min - minimum; *Max – maximum; - no standard limits classified by ASTM standard

4.7 Pellet morphology

During the verification process, the pellet morphology for maximising levels of biomass and lipid for *G. lucidum* was observed both macroscopically and microscopically, as depicted in Figure 4.5. The findings indicate that the shape of mycelial pellets varies under different cultivation parameters, with each response favouring distinct morphology characteristics. In Figure 4.5a, the formation of small and starburst-like globular pellets with loosely branching outer layer were observed. This result aligns with literature reports, where small-compact pellets are observed to favour high biomass production (Abdullah et al., 2020; Supramani et al., 2019b). Figure 4.5b shows the formation of clumped and irregular-shaped pellets with smooth hairy surface. This morphology suggests reduced surface friction, which may facilitate interaction with surrounding medium and potentially increase lipid production (Gao et al., 2014). Previous research indicates that stress influences pellet morphology, causing mycelium aggregation into pellet formations, possibly through self-immobilization. This clumping creates shield for fungi within the liquid medium, and is consequently linked to enhanced lipid production (Veiter et al., 2018). Furthermore, Figure 4.5c depicts that culture conditions optimizing both biomass and total lipid production tend to favour larger-sized pellets with the formation of starburst-like and thick-branched structures.

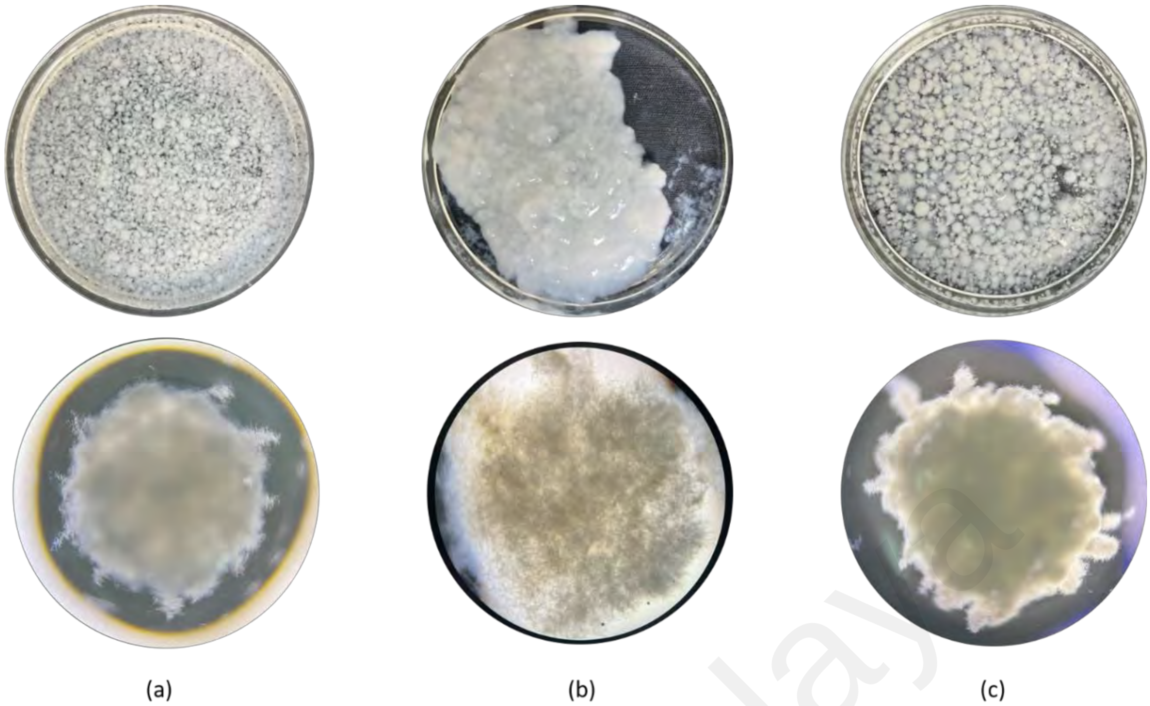

Figure 4.5: Macroscopic (above) and microscopic (below) (magnified at 4 x under microscope) for morphological observation of mycelium pellets of *G. lucidum* under optimized fermentation parameters for responses (a) biomass, (b) total lipid, and (c) biomass and total lipid.

4.8 Comparative analysis of current study with literature

A comparison of the production of biomass and lipid using submerged liquid fermentation between different *G. lucidum* species is shown in Table 4.7. The biomass and lipid yield from *G. lucidum* vary depending on the strain, although the application of RSM led to increased production of *G. lucidum* products in all optimization studies. To my understanding, no previous research has focused on optimizing the media compositions and fermentation parameters for lipid production in *G. lucidum*. The data presented in this study utilized RSM, providing insights into the experimental parameters for lipid production of *G. lucidum*.

Table 4.7: The literature comparison for the optimization for *G. lucidum* using submerged liquid fermentation.

Species	Optimization method	Cultivation method	Working volume (mL)	Initial pH	Glucose concentration (g/L)	Agitation (rpm)	Biomass (g/L)	EPS (g/L)	ENS (g/L)	Lipid Yield (% w/w)	Reference
G. lucidum QRS 5120	RSM	Shake Flask ALSB	200 2000	6	50	113	8.33 5.32	NA	NA	2.17 2.35	Current study
G. lucidum QRS 5120	RSM	ALSB	2000	4	30	110	7.90	4.60	NA	NA	(Supramani et al., 2023)
G. lucidum QRS 5120	RSM	Shake Flask	200	4	26.5	100	5.19	2.64	1.52	NA	(Supramani et al., 2019a)
G. lucidum BGF4A1	RSM	Shake Flask	200	5.26	50	-	3.12	1.96	NA	NA	(Hassan et al., 2019)
G. lucidum-0201	One-factor-at-a-time (OFAAT)	Shake Flask	100	5	15	-	3.68	NA	NA	NA	(Shah & Modi, 2018)
G. lucidum CAU5501	OFAAT and orthogonal matrix method	Shake Flask	1200	-	50	150	7.235	1.723	NA	NA	(Yuan et al., 2012)

CHAPTER 5 RELATION WITH SDG 7 AFFORDABLE AND CLEAN ENERGY, AND SDG 13 CLIMATE ACTION

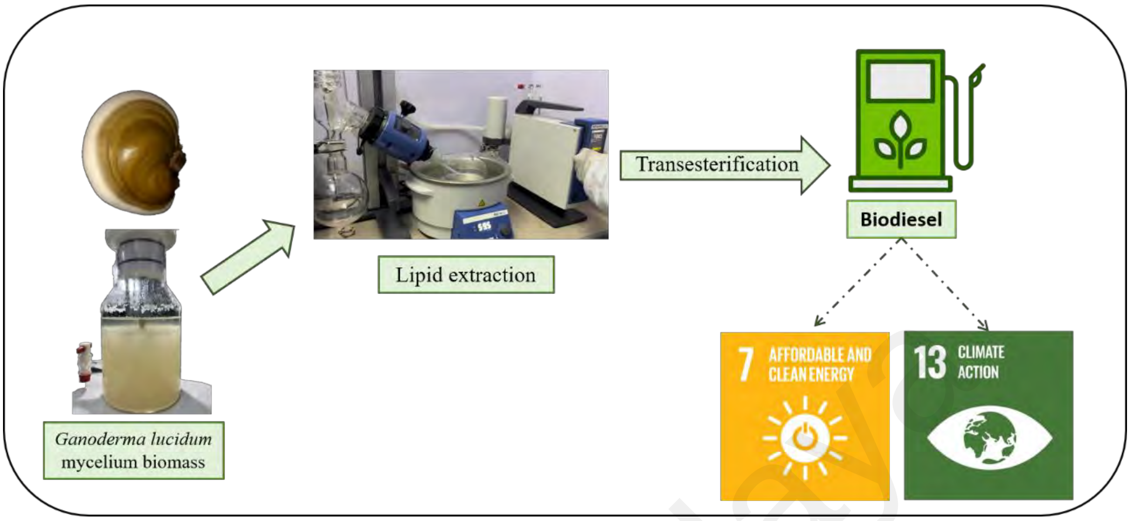

Figure 5.1: The contribution of *G. lucidum*-derived biodiesel to SDGs.

Based on my study, it can be observed that *G. lucidum* exhibits the capability for lipid production through liquid fermentation. This study is relevant in the framework of current trends towards achieving Sustainable Development Goals (SDGs). The keys SDGs impacted by the lipid-producing capability of *G. lucidum* through liquid cultivation are SDG 7 and SDG 13, as shown in Figure 5.1. By demonstrating the transformation of *G. lucidum* lipids into biodiesel, this study contributes to the goals of SDG 7, which aims to facilitate universal access to affordable, reliable, sustainable, and modern energy for all. Lipids produced from *G. lucidum* can potentially exhibit as a novel renewable and sustainable feedstock for biodiesel production, thereby promoting clean energy alternatives to fossil fuels. This bolsters efforts towards energy diversification and reduces reliance on non-renewable energy sources, thereby contributing to the development of sustainable energy.

Moreover, the study of *G. lucidum* biomass as a biodiesel feedstock is in accordance with the objectives of SDG 13, which seek to address climate change and its consequences. Fungi-based biodiesel production offers an environmentally sustainable alternative to conventional fossil fuels, which contribute significantly to the emissions of greenhouse gases and global warming. By harnessing fungi biomass for biodiesel production, there is an opportunity to diminish dependence on exhaustible fossil fuel resources and shift towards sustainable energy sources. This development not only helps combat climate change by mitigating greenhouse gas emissions but also promote environmental sustainability.

In summary, the research findings regarding the lipid production and biodiesel conversion derived from *G. lucidum* mycelium biomass have important implications for advancing both SDG 7 and SDG 13. By highlighting the renewable energy and climate mitigation potential of *G. lucidum*-derived biodiesel (Ganodiesel), this study contributes to global efforts to achieve sustainable development and address pressing environmental challenges.

CHAPTER 6 CONCLUSION

This study presented the optimized fermentation parameters for *G. lucidum* biomass and lipid production using RSM. The glucose concentration and initial pH significantly influenced biomass production, while glucose concentration, initial pH and agitation had a significant effect on lipid production. Under optimized conditions of an initial medium pH 6, 50 g/L glucose, and agitation at 113 rpm, the results showed a biomass of 5.32 g/L and a total lipid yield of 2.35% in ALSB. In shake flasks, the biomass reached 8.33 g/L with a total lipid yield of 2.17%. Furthermore, the analysis of *G. lucidum* lipid revealed a significant composition of palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2) at 18.60%, 16.21%, and 48.11%, respectively. The predominant fatty acids identified in *G. lucidum* lipid are crucial for biodiesel production and closely align with those present in commercial vegetable oils used for this purpose. Moreover, the biodiesel properties of Ganodiesel, except density were in accordance with ASTM D6751-08 and EN 14214 standards. Therefore, this study provides a groundwork for the potential lipid production of liquid-cultivated *G. lucidum* mycelium and its application in the field of bioenergy. Lipid produced from *G. lucidum* mycelium can potentially serve as a renewable and sustainable source for biodiesel production, thereby promoting clean energy alternatives to fossil fuels.

In terms of future perspectives, further research could focus on further enhancing the process by employing the optimum parameters of *G. lucidum* growth in ASLB and associated with the optimization of lipid production to maximize both biomass and lipid in ALSB. Additionally, there is an opportunity to refine lipid extraction techniques, including the selection of solvents and the ratio, to improve overall efficiency. These research directions could help advance the practical utilization of *G. lucidum* in bioenergy, thereby supporting efforts towards achieving SDG 7 and SDG 13.

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