OPTIMISATION OF LIPID AND CARBOHYDRATE SYNTHESIS IN *Tetradesmus obliquus* UPSI-JRM02 UNDER NITROGEN STRESS CONDITION FOR BIOFUEL FEEDSTOCK PRODUCTION

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UNIVERSITI PENDIDIKAN SULTAN IDRIS

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ABSTRACT

The optimisation of lipid and carbohydrate yields and productivity is important for enhancing microalgae biofuel production. This study aims to optimise the growth conditions of *Tetradesmus obliquus* UPSI-JRM02 for high lipid and carbohydrate production and to examine the synthesis of these biomolecules under nitrogen stress conditions. The optimisation study was carried out using response surface methodology with Design Expert software. Four variables (nitrogen concentration, temperature, pH, and light intensity) were optimised to enhance *T. obliquus* productivity. *T. obliquus* was then cultured in optimised conditions before being introduced in nitrogen stress condition to further increases the production of lipid and carbohydrate yields. The results indicated that the highest lipid (33 mg/L/day), carbohydrate (51 mg/L/day) and biomass (115 mg/L/day) productivity were produced at a nitrogen concentration of 400 mg/L NO_3^- at 36 ^oC with pH of 9.8, and light intensity of 23,500 lux. The lipid and carbohydrate yields obtained under optimised conditions were 24% and 41%, respectively. Meanwhile, the highest lipid and carbohydrate yields achieved under nitrogen stress conditions were 36.5% and 23%, respectively. These results suggested carbon was shifted into lipid synthesis under nitrogen stress condition. The fatty acid methyl ester synthesised under nitrogen stress condition was comprised of 54.07% polyunsaturated fatty acid, 43.33% saturated fatty acid, and 2.6% monounsaturated fatty acid. The fluorescence and transmission electron microscopy images further confirmed the presence of lipid bodies and starch granules in *T. obliquus.* As a conclusion, the yield and productivity of lipid and carbohydrate of *T. obliquus* UPSI-JRM02 had sucessfully increased at the optimum conditions. Meanwhile, the nitrogen stress condition improved only the lipid yield of *T. obliquus*. The implication of this study shows the potential of *T. obliquus* UPSI-JRM02 to produce promising biofuel feedstock for future application.

PENGOPTIMUMAN SINTESIS LIPID DAN KARBOHIDRAT PADA KEADAAN STRES NITROGEN OLEH *T. obliquus* UPSI-JRM02 BAGI PENGHASILAN STOK SUAPAN BAHAN BAKAR BIO

ABSTRAK

Pengoptimuman hasil dan produktiviti lipid dan karbohidrat penting untuk meningkatkan penghasilan bahan bakar bio mikroalga. Kajian ini bertujuan untuk mengoptimumkan keadaan pertumbuhan *Tetradesmus obliquus* UPSI-JRM02 untuk penghasilan lipid dan karbohidrat yang tinggi dan untuk mengkaji sintesis biomolekul ini dalam keadaan stres nitrogen. Kajian pengoptimuman dilakukan dengan menggunakan *response surface methodology* dengan perisian *Design Expert*. Empat pembolehubah (kepekatan nitrogen, suhu, pH dan keamatan cahaya) dioptimumkan untuk meningkatkan produktiviti *T. obliquus*. *T. obliquus* kemudiannya dikultur dalam keadaan optimum sebelum diberi keadaan stres nitrogen untuk meningkatkan lagi hasil lipid dan karbohidrat. Dapatan kajian menunjukkan bahawa lipid tertinggi (33 mg/L/hari), karbohidrat (51 mg/L/hari) dan produktiviti biojisim (115 mg/L/hari) dihasilkan pada kepekatan nitrogen 400 mg/L NO_3^- pada 36 ºC dengan pH 9.8, dan keamatan cahaya sebanyak 23,500 lux. Hasil lipid dan karbohidrat yang diperolehi di bawah keadaan optimum masing-masing adalah 24% dan 41%. Sementara itu, hasil lipid dan karbohidrat tertinggi yang dicapai di bawah keadaan stres nitrogen masing-masing adalah 36.5% dan 23%. Hasil kajian ini mencadangkan bahawa agihan karbon beralih kepada sintesis lipid di bawah keadaan stres nitrogen. Asid lemak metil ester yang disintesis di bawah keadaan stres nitrogen terdiri daripada 54.07% asid lemak poli tak tepu, 43.33% asid lemak tepu dan 2.6% asid lemak mono tak tepu. Imej pendarfluor dan mikroskopi elektron transmisi selanjutnya mengesahkan kehadiran jasad lipid dan granul kanji dalam *T. obliquus*. Sebagai kesimpulan, hasil dan produktiviti lipid dan karbohidrat *T. obliquus* UPSI-JRM02 telah berjaya ditingkatkan pada keadaan optimum. Manakala keadaan stres nitrogen hanya meningkatkan hasil lipid *T. obliquus*. Implikasi kajian ini menunjukkan potensi *T. obliquus* UPSI-JRM02 sebagai stok suapan bahan bakar bio untuk aplikasi masa hadapan.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
CCD	Central composite design
CFPP	Cold filter plugging point
CN	Cetane number
DoE	Design Expert
DU	Degree of unsaturation
FAME	Fatty acid methyl easter
HHV	High heating value
IV	Iodine value
KV	Kinematic velocity
LCSF	Long-chain saturated factor
MUFA	Monounsaturated fatty acid
NADP	Nicotinamide adenine dinucleotide phosphate
OD	Optical Density
PUFA	Polyunsaturated fatty acid
RSM	Response surface methodology
SFA	Saturated fatty acid
SV	Saponification value
TAG	Triacylglycerides
TEM	Transmission electron microscope

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CHAPTER 1

INTRODUCTION

1.1 Research Background

The main resources for energy currently are petroleum, coal and natural gas. Demand is still rising; however, there has been an increase in cost due to diminishing stocks of fossil fuels. This current trend in fossil fuel consumption has prompted interest in developing alternative energy sources that are renewable, have more competitive production costs, reduce greenhouse gas emissions and represent greener biofuel products (Brennan & Owende, 2012). Generally, unlike the ancient deposits of fossil fuels, biofuels are energy-rich compounds based on biomass produced from living organisms or microorganisms that can be regenerated in a relatively short period of time (Voloshin, Rodionova, Zharmukamedov, Veziroglu & Allakhverdiev, 2016).

To date, the production of biofuels can be viewed as having evolved from first to third generation sources. First-generation biofuels are derived from edible crops, such as corn, wheat, sugarcane and barley, which are unsustainable due to competition from their use as food commodities, and which also produce a carbon footprint that increases with larger cultivation area (Scaife, Merkx-Jacques, Woodhall & Armenta, 2015). Second-generation biofuels are produced from lignocellulosic biomass of non-edible crops, which rely on pre-treatment by chemicals (Naik, Goud, Rout & Dalai, 2010). Meanwhile, third-generation biofuels are produced from photosynthetic microorganisms, such as cyanobacteria and microalgae, which are renewable and carbon neutral, and thus have the potential to meet global energy demands (Sarkar & Shimizu, 2015).

Algae can be categorised as macroalgae or microalgae according to their morphological features and size. Macroalgae usually refer to seaweeds, which are multicellular primitive plants with a size in the range of 0.001–2 mm (Suparmaniam, Lam, Uemura, Lim, Lee & Shuit, 2019). By contrast, microalgae are unicellular photosynthetic organisms that contain chlorophyll pigment for energy transformation. They consist of four main groups, including the Cyanophyceae (blue-green algae), Chlorophyceae (green algae), Bacillariophyceae (diatoms) and Chrysophyceae (golden algae). Members of the Cyanophyceae are prokaryotic, while the latter three groups are eukaryotic microalgae (Suparmaniam et al., 2019). Microalgae have simple cell proliferation and reproductive systems, enabling them to propagate in a variety of habitats, including freshwater (Rajkumar & Takriff, 2016), and harsh environments, such as polar snow (Hulatt, Berecz, Egeland, Wijffels, & Kiron, 2017), those with high temperatures (Patel, Matsakas, Rova, & Christakopoulos, 2019), high salinity and alkaline soils (Qiao, Takano & Liu, 2015) and landfill leachates (Nordin, Yusof & Samsudin, 2017).

Ramaraj (2015) stated that microalgae are promising biofuel resources for the future, particularly due to the advantages of using microalgal feedstock over traditional feedstocks from plant biomass. Microalgae show rapid growth, thus enabling many species to produce high lipid contents of 20–50% biomass dry weight (Suparmaniam et al., 2019). They are also able to produce ten times more oil compared to plants, due to their higher lipid content and biomass. In addition, cultivation of microalgae can be performed using wastewater with high nitrogen contents, such as for ammonium and nitrate (Nordin, Yusof & Samsudin, 2017) thus helping to reduce water pollution. With regard to air quality control, carbon fixation through microalgae photosynthesis enables carbon sequestration of greenhouse gases. Microalgae, which occur in aquatic ecosystems, lack structural components such as lignocellulose, hemicellulose and lignin, thus their biomass is predominantly lipid, carbohydrate and protein.

There were many microalgae strains reported to be capable of producing lipid, such as *Botryococcus braunii*, *Chlamydomonas pitschmannii*, *Chlorella vulgaris* and *Nannochloropsis* sp., with lipid content ranges of 25–75%, 51%, 5–58% and 12–53%, respectively (Fulbright et al., 2018). Carbohydrate content in microalgae varies according to species, such as in *Chlorella vulgaris* (37–55%; Suparmaniam et al., 2019), *Chlamydomonas reinhardtii* (9.2%; Parsa, Jalilzadeh, Pazoki, Ghasemzadeh, & Abduli, 2018) and C*hlorococum* sp. (32.5%; Suparmaniam et al., 2019). Microalgae biomass, particularly lipid and carbohydrate, is useful as a feedstock for the production of biodiesel, bioethanol, biohydrogen and bio-oil (Lee, Seong, Lee & Lee, 2015).

Although the production of microalgal biofuels has been widely investigated and commercialised, large-scale production is still hindered by high production costs. Therefore, current research endeavours to search for appropriate microalgae strains, and to optimise biomass, lipid and carbohydrate production, as well as maximising the value of microalgae by-products.

Microalgae growth depends on various factors, such as nitrogen sources, nitrogen concentrations, light intensity, temperature, pH, CO_2 concentrations and salinity. These factors influence growth rates, the composition of biomolecules, such as carbohydrate, lipids and protein, and the overall productivity of microalgae. However, microalgae can be exposed to various environmental factors that resulted in changes to their physiology, growth response and the metabolic pathways of lipids and carbohydrates. Even so, several microalgae strains have been reported to survive under harsh conditions, such as in landfill leachates and agro-industrial wastewater, and still maintain high growth rates and lipid contents (Milano et al., 2016; Nordin, Yusof & Samsudin, 2017; Markou, Angelidaki & Georgakakis, 2012). Notably, microalgae that are cultured under stress conditions, such as nitrogen limitation, have the ability to change their biomass composition and generate higher levels of lipids than carbohydrates (Markou & Nerantzis, 2013).

Tetradesmus obliquus, previously classified as *Scenedesmus obliquus*, is characterised by single ellipsoidal cells or colonial green algae with four coenobia arranged in a row (Pancha et al., 2014). Recently, cultivation of *T. obliquus* has received considerable interest due to its relatively high growth rate, tolerance for wastewater and significant accumulation of lipids (Esakkimuthu, Wang, El-Fatah Abomohra, Shanmugan & Ramakrishnan, 2019). However, the accumulation of lipids and carbohydrates by *T. obliquus* is varied, and greatly influenced by environmental factors. Previous studies have reported that lipid content for the Scenedesmaceae family, including *T. obliquus*, can be maximised to 39–44% under nitrogen-deficient conditions (Valdez-Ojeda et al., 2015; Mandal & Mallick, 2009). For these studies, optimisation and synthesis of lipid and carbohydrates employed a locally isolated microalgae strain *T. obliquus* UPSI-JRM02 (Nordin, Yusof & Samsudin, 2014).

With reference to the aforementioned issue, optimisation of cell cultivation in various culture conditions is necessary to maximise the yield and productivity of microalgae. To date, various methods have been used in optimisation studies, such as a classical design of experiments, the Taguchi method, and response surface methodology (RSM), a statistical tool allowing multivariable optimisation studies with a reduced number of experimental runs and involving less time. In addition, RSM based on the central composite design (CCD) has the advantage of predicting responses based on only a few sets of experimental data, in which all the variables vary within a selected range (Silva, Camargo & Ferreira, 2011). Optimisation studies are beneficial for improving the economic feasibility of microalgae biofuel production.

1.2 Problem Statement

1.2.1 Global Warming and Climate Change Issues

Currently more than 80% of the energy supply comes from coal, natural gas and petroleum, thus causing substantial emissions of air pollutants, such as carbon dioxide, carbon monoxide, nitrogen oxide, particulate matter and polycyclic aromatic hydrocarbons (PAHs) (Yan et al., 2019). As such, the problems of global warming and climate change are mainly attributed to over consumption of fossil fuels. Because of their origin, fossil fuels are considered non-renewable and not sustainable, since they cannot be replenished in a short period of time. The burning of fossil fuels results in emission of carbon dioxide and other greenhouse gases, causing heat to be trapped in the atmosphere, which contributes to global warming and climate change. Therefore, there is an urgent need for renewable and sustainable energy supplies to be developed, due to dwindling reserves of fossil fuels and their impact on global climate. Among the potential renewable energy sources that have recently generated interest is microalgae, creating emerging opportunities to replace current non-renewable energy sources (Khan et al., 2018).

The advantages of microalgae feedstock for energy production include: (1) high growth rate and productivity; (2) lack of hemicelluloses and lignin content requiring pre-treatment; (3) efficient consumption of carbon dioxide helping to reduce greenhouse gas emissions; (4) ability to grow in wastewater, thereby treating the wastewater and reducing freshwater use; and (5) they can be harvested in a shorter period of time (1–10 days) than other feedstocks, thus providing sufficient supplies to meet biofuel production demands (Chen et al., 2013). Therefore, cultivation of *T. obliquus* is imperative to explore its potential in generating biofuel feedstock and to address non-renewable energy consumption issues.

1.2.2 First and Second Generation Biofuel Issues

First generation biofuels refer to those extracted from food sources, either plants or animals. This type of biofuel was developed due to the urgent demand to replace exhausted fossil fuel resources. Edible crops, such as corn, wheat, potato, barley and sugarcane are capable of producing biodiesel, bioethanol and biogas (Alalwan, Alminshid, & Aljaafari, 2019). However, utilisation of edible biomass as feedstock for biofuels is controversial, due to its impact on food supply, biodiversity and agriculture land. The utilisation of edible biomass feedstock competes with food supply, thus requiring more land for crops. In addition, opening more land for agriculture contributes to deforestation and loss of biodiversity. Intensive farming activities may also trigger soil degradation and increase pesticide use (Suparmaniam et al., 2019).

Due to these issues, second generation biofuels were established to maximise the use of waste biomass, mainly from plants (Alalwan et al., 2019). Lignocellulosic biomass from plant waste is an inexpensive and abundant biofuel feedstock, and an attractive new option for fossil fuel substitution. However, cost effectiveness of second generation biofuel production is still hindered by several limitations. Feedstock from waste biomass is characterised by a complex composition of lignin, cellulose and hemicellulose; therefore, pre-treatments, such as a two stage acid hydrolysis (Chin, Lim, Pang, Lim, & Lee, 2019) and use of a deep eutectic solvent (Thi & Lee, 2019) are required in order to obtain high feedstock yields, incurring cost to the production of biofuel. Because of the disadvantages associated with first and second generation biofuels, third generation biofuels from microalgae biomass have been explored.

1.2.3 Microalgal Biofuel Feedstock Production is Strain Specific and Dependent on Culture Conditions

The economic feasibility of microalgal mass culture is determined by cultivating the appropriate microalgae species that shows high lipid and carbohydrate production. Although microalgae have high biomass productivity, the production of microalgae biofuel actually suffers from high capital and operating costs due to low lipid and carbohydrate yield and productivity (Tan & Lee, 2016). Therefore, selection of an appropriate strain is crucial prior to the production of the biofuel. The production of lipid content from various species of microalgae was in the range of 5–75% (Fulbright et al., 2018). However different microalgae species exhibited varying lipid content when cultivated under different culture conditions. For example, *T. obliquus* BR003 and *T. bajacalifornicus* BR024 had 6.5% and 7% lipid contents, respectively, when grown under salt stress and a semi continuous mode of culture (Rocha et al., 2019). Meanwhile, 48.12% lipid content was achieved for *T. abundans* when cultured in Fogg's medium in the absence of nitrogen (Rai & Gupta, 2017).

In addition to choosing a suitable microalgae strain, growth conditions, such as nutrient requirements, temperature, light intensity, pH and carbon dioxide concentration influence lipid and carbohydrate yields in microalgae. The microalgae response to culture conditions, particularly environmental stresses, is varied, producing different lipid and carbohydrate contents. Environmental stress conditions such as nitrogen limitation, light intensity and salinity are capable of increasing lipid yield (Chen et al., 2017). Therefore, manipulation of these factors through optimisation of the culture conditions is essential to enhance feedstock yield.

To date, a number of optimisation studies have been conducted to improve the lipid and carbohydrate yield of *T. obliquus* under various environmental conditions, including nitrogen stress (Martin et al., 2014). Under nitrogen stress, lipid is accumulated due to catabolism of amino acids that produces various tricarboxylic acid (TCA) cycle intermediates, such as acetyl coA for fatty acid elongation (Chellamboli & Perumalsamy, 2014; Martin et al., 2014; Yang et al., 2014). However, previous studies of *Tetradesmus* sp. have mainly focused on lipid production alone, instead of both carbohydrates and lipids (Chellamboli & Perumalsamy, 2014; Yang et al., 2014). By contrast, this study focuses on both macromolecules, which is crucial as these biomolecules share the same fixed carbon precursor from photosynthesis. In addition, changes of lipid and carbohydrate yields under nitrogen stress are important to understand in exploiting both biomolecules for the future production of biofuel feedstocks.

In conclusion, overconsumption of fossil fuel which resulted in emission of greenhouse gaseous has led to the emergence of microalgae biofuel. Furthermore, the third generation microalgae biofuel does not compete with food resources as demonstrated by the first generation biofuel. In contrast to second generation biofuel, microalgae biofuel is less expensive as no pre-treatment cost incurred in its production. Therefore, optimisation of lipid and carbohydrate in *T.obliquus* UPSI-JRM02 using RSM was conducted to enhanced biofuel production.

1.3 Significance of the Study

1.3.1 Renewable Energy and Carbon Sequestration

Microalgae as biofuels are capable of replacing depleted fossil fuel resources, which are not renewable. They offer an interesting photosynthetic ability, being able to generate biochemical compounds, such as lipids and carbohydrates, that can act as biofuel feedstocks (Sarkar & Shimizu, 2015). The carbon dioxide consumption by microalgae through photosynthesis allows atmospheric carbon sequestration, thus reducing global warming. Furthermore, exploring local microalgae potential, such as for *T. obliquus*, refines its application and technology requirements to meet future local needs.

1.3.2 Optimisation of Microalgae Culture Conditions to Increase Lipid and Carbohydrate Yields

Great progress in microalgae biofuels have been made in the past few years in improving lipid and carbohydrate yields. This study was designed to increase lipid and carbohydrate production by manipulating the growth conditions of microalgae, including nitrogen concentrations, temperature, light intensity and pH. The RSM, which involves mathematical and statistical techniques, has been widely applied in the optimisation of processes and products, including those in biotechnology and the food industries (Mandenius, 2008). The RSM is useful, as it provides an understanding of the effect of selected variables on process responses, interactions between selected variables and the combination of effects that all the selected variables have on the process responses. The optimum conditions for microalgae and maximum yield are necessary prior to scale-up of the biofuel feedstock process for commercial applications.

1.3.3 Cost-Effective Microalgae Cultivation under Nitrogen Stress Conditions

The cost of microalgae biofuel is high considering the need to provide nutrients. As such, by reducing one of the main variables for microalgal growth, production costs might be reduced. Nitrogen is an essential element for microalgae growth, as it is used to synthesise nucleic acids, chlorophyll and amino acids. Chen et al. (2017) showed that biosynthesis and accumulation of lipids is higher under nitrogen stress conditions for microalgae of various taxonomic groups. According to Griffiths, van Hille & Harrison (2014), nitrogen stress is the most frequently method used to increase lipid production, as it is easier to manipulate and has a large influence on lipid content of microalgae. Nitrogen stress not only reduces the cost of culture media, but also enhances lipid and carbohydrate yields for both biodiesel and bioethanol feedstocks, respectively.

1.4 Research Hypotheses

This study was designed to evaluate the following research hypotheses:

1) Optimisation of *T. obliquus* UPSI-JRM02 growth conditions using RSM could increase lipid and carbohydrate yield and productivity.
2) Nitrogen stress conditions could increase *T. obliquus* lipid and carbohydrate yield and productivity.

1.5 Objectives

Based on the aforementioned problem statements, this study was devised to achieve the following objectives:

1) To optimise the growth conditions of *T. obliquus* UPSI-JRM02 for high lipid and carbohydrate yields and productivity by using RSM.
2) To examine the synthesis of lipid and carbohydrate under nitrogen stress conditions.

1.6 Scope and Limitations of the Study

In order to achieve the aforementioned objectives, several scopes of the experiments were defined, and limitations were identified:

1) This study was conducted using a local microalgae species, *T. obliquus* UPSI-JRM02. This species was isolated from a landfill leachate treatment plant and

tolerated high N-NO_3^- concentrations up to 1500 mg/L (Nordin, Samsudin & Yusof, 2017).

2) Optimisation of the growth conditions were performed using RSM with Central Composite Design (CCD). The variables selected were nitrogen concentration, temperature, pH, and light intensity, with their ranges being based on previous studies (Ren, Liu, Ma, Zhao & Ren., 2013; Liu et al., 2012). Thirty sets of experimental runs were performed on a laboratory scale in 500 ml Erlenmeyer flasks.

3) Cultivation of *T. obliquus* was performed in a 2.0 l photobioreactor to examine the synthesis of lipid under nitrogen stress conditions. *T. obliquus* was cultured under optimum conditions determined in the optimisation study, and in the absence of a nitrogen source. Lipid, carbohydrate and protein yields and productivity under both conditions were monitored and compared.

1.7 Organisation of the Thesis

This thesis focused on the optimisation of growth conditions for *T. obliquus* using RSM and examined the synthesis of lipid and carbohydrate under nitrogen stress conditions. The thesis consists of five chapters (Figure 1.1). Chapter 1 contains research background, problem statements, the significance of the study, research hypotheses, research objectives and the scope of the study. Chapter 2 reviews the literature on *T. obliquus*, the microalgae species used in this study, the factors that affect the growth of *Tetradesmus* spp., lipid and carbohydrate synthesis in microalgae, carbon partitioning under nitrogen stress conditions, the performance and production of biomass, the use of microalgae as sources of biofuel, and previous optimisation studies. Chapter 3 describes the materials and methods used in this study.

Chapter 4 contains the results and discussion for this study. It is divided into two parts, (1) the optimisation of *T. obliquus* using RSM, and (2) the synthesis of lipid and carbohydrate under nitrogen stress conditions. The optimisation and nitrogen stress experiments were carried out at a laboratory scale. Thirty sets of experiments were performed with the selected variables of nitrogen concentration, temperature, pH and light intensity. Each set of the experiment took about 2–3 weeks, including the culturing process and biochemical analysis. The synthesis of lipid and carbohydrate under nitrogen stress conditions was assessed using a 2 L bioreactor. This study involved a two-step process, (i) microalgae culture conditions determined in the first part of the study, followed by (ii) microalgae culture in nitrogen stress conditions. Samples were subject to biochemical analysis, visualisation of lipid bodies using fluorescence microscopy, and observation of starch granules and lipid bodies by transmission electron microscopy (TEM). Chapter 5 contain the conclusion of this study and recommendation for the future study.

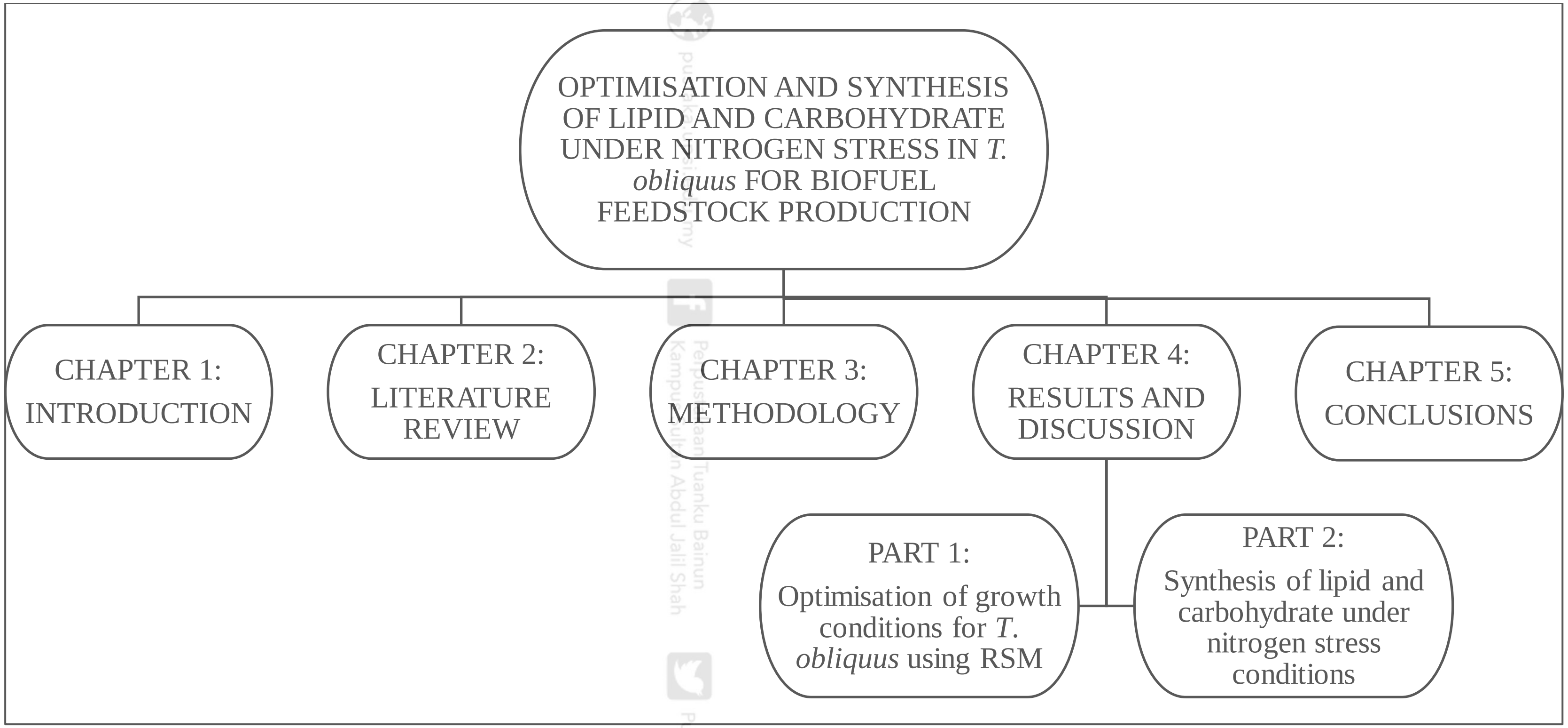

Figure 1.1. Overview of the thesis "Optimisation and Synthesis of Lipid and Carbohydrate under Nitrogen Stress in *T. obliquus* for Biofuel Feedstock Production"

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