# OPTIMISATION OF BIOMASS AND LIPID PRODUCTION OF A TROPICAL THRAUSTOCHYTRID *Aurantiochytrium* sp. UMACC-T023 IN SUBMERGED-LIQUID FERMENTATION FOR LARGE-SCALE BIODIESEL PRODUCTION

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**2020**

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**OPTIMISATION OF BIOMASS AND LIPID PRODUCTION OF A TROPICAL THRAUSTOCHYTRID *Aurantiochytrium* sp. UMACC-T023 IN SUBMERGED-LIQUID FERMENTATION FOR LARGE-SCALE BIODIESEL PRODUCTION**


## ABSTRACT

A tropical thraustochytrid, *Aurantiochytrium* sp. UMACC-T023, was optimised for high biomass and lipid production in submerged-liquid fermentation (SLF). Biomass and lipid production were optimised based on glucose, polypeptone, and yeast extract concentration using response surface methodology (RSM). In RSM, the applied central composite design (CCD) showed that the optimisation model was significant for all variables studied. Yeast extract and polypeptone were associated with significant effects in UMACC-T023 biomass production with $p < 0.05$. The model was validated by employing the optimised media composition in shake flasks and in 1.4-L stirred-tank bioreactors. The optimised media composition for both biomass and lipid production was 10 g $L^{-1}$ glucose, 15.08 g $L^{-1}$ polypeptone, and 13.56 g $L^{-1}$ yeast extract. Biomass production in the bioreactor increased 2.12-fold compared with the shake flask culture utilising the same optimised media composition. This result demonstrated that the optimized media composition can be utilised for large-scale lipid production of UMACC-T023 for potential biodiesel feedstock as it also fulfilled the international standards for biodiesel.



**Keywords**: *Aurantiochytrium* sp., biomass, biodiesel, response surface methodology, submerged-liquid fermentation

**OPTIMISASI PENGHASILAN BIOJISIM DAN LIPID DARIPADA THRAUSTOCHYTRID TROPIKAL *Aurantiochytrium* sp. UMACC-T023 DI DALAM FERMENTASI CECAIR TERENDAM UNTUK PENGHASILAN BIODIESEL PADA SKALA YANG BESAR**

## ABSTRAK

Satu thraustochytrid tropikal, *Aurantiochytrium* sp. UMACC-T023 telah dioptimisasi untuk penghasilan biojisim dan lipid yang tinggi di dalam fermentasi cecair terendam. Penghasilan biojisim dan lipid telah dioptimisasi berdasarkan kepekatan glukosa, polipepton dan ekstrak yis menggunakan kaedah 'response surface methodology' (RSM). Di dalam kaedah RSM ini, 'central composite design' (CCD) telah digunakan dan menunjukkan yang model optimisasi adalah signifikan bagi semua pembolehubah yang dikaji. Ekstrak yis dan polipepton telah dikaitkan dengan kesan yang signifikan dalam penghasilan biojisim oleh UMACC-T023 dengan nilai $p < 0.05$. Model tersebut telah disahkan dengan menggunakan komposisi media yang telah dioptimum di dalam fermentasi kelalang konikal dan di dalam 1.4 L bioreaktor tangki teraduk. Komposisi media yang dioptimum untuk penghasilan biojisim dan lipid ialah 10 g $L^{-1}$ glukosa, 15.08 g $L^{-1}$ polipepton dan 13.56 g $L^{-1}$ ekstrak yis. Penghasilan biojisim di dalam bioreaktor meningkat 2.12 kali ganda berbanding dengan di dalam fermentasi kelalang konikal apabila menggunakan komposisi media dioptimum yang sama. Hasil kajian ini menunjukkan yang komposisi media yang dioptimum boleh dimanfaatkan untuk penghasilan lipid oleh UMACC-T023 pada skala yang besar dan juga menunjukkan potensi sebagai bahan mentah biodiesel kerana ianya turut memenuhi standard antarabangsa bagi biodiesel.

**Kata kunci:** *Aurantiochytrium* sp.; 'response surface methodology'; fermentasi cecair terendam; biojisim; biodiesel

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | | |
|---|---|---|
| °C | : | Degree Celsius |
| ANOVA | : | Analysis of variance |
| C:N | : | Carbon to nitrogen ratio |
| CCD | : | Central composite design |
| CN | : | Cetane number |
| CSL | : | Corn steep liquor |
| DHA | : | Docosahexaenoic acid |
| DO | : | Dissolved oxygen |
| DW | : | Dry weight |
| EPA | : | Eicosapentaenoic acid |
| FAS | : | Fatty acid synthase |
| g | : | Grams |
| GYP | : | Glucose-yeast extract-polypeptone |
| h | : | Hour |
| ID | : | Ignition delay |
| kg | : | Kilogram |
| kLa | : | Volumetric mass transfer coefficient |
| L | : | Litres |
| mg | : | Milligrams |
| mL | : | Millilitres |
| MSG | : | Monosodium glutamate |
| MUFA | : | Monounsaturated fatty acid |
| nm | : | Nanometres |
| OD | : | Optical density |

| | | |
|---|---|---|
| OFAAT | : | One-factor-at-a-time |
| OTR | : | Oxygen-transfer-rate |
| PKS | : | Polyketide synthase |
| PUFA | : | Polyunsaturated fatty acid |
| RPM | : | Revolutions per minute |
| RSM | : | Response surface methodology |
| SD | : | Standard deviation |
| SF | : | Shake flasks |
| SFA | : | Saturated fatty acid |
| sp. | : | Species |
| STB | : | Stirred-tank bioreactors |
| TFA | : | Total fatty acid |
| vvm | : | Volume of gas per volume of liquid per minute |
| β | : | Beta |

# CHAPTER 1: INTRODUCTION

## 1.1 Research background

Increasing energy usage for transportation and industrialisation signifies a greater demand on energy supply (Chu & Majumdar, 2012). At present, energy supplies are primarily generated from fossil fuels. However, fossil fuels are non-renewable and are showing signs of depletion, and the use of biofuels such as biodiesel and bio-ethanol may represent a solution to this problem (Mata *et al.*, 2010). However, the use of biofuel to replace fossil fuels is not without limitations, which are mostly related to the source from which the biofuel is obtained. First-generation biofuels have been generated from crop plants, which also serve as a food supply for human consumption. This issue leads to research on producing biodiesel using oils from inedible seed such as sweet basil seed (Amini *et al.*, 2017). The extensive use of food crops as a source of biofuel can also lead to an increase in land use for plantation, which can lead to biodiversity loss (Mata *et al.*, 2010). Second-generation biofuels are generated from plant or animal biomasses, which are more sustainable, although the lipid extraction process is more challenging. Researchers have subsequently turned their focus to third-generation biofuels generated from microalgae biomasses such as methane, biodiesel, and biohydrogen (Chisti, 2007). Open or closed cultivation systems such as raceways and enclosed photobioreactors can be used for microalgae cultivation and their biomasses can be harvested for lipid extraction and conversion into biodiesel (Yew *et al.*, 2019). Open cultivation systems have demonstrated lower operation costs, but it is more difficult to control the quality of the biomass and to maintain production stability (Yew et al., 2019). Microalgae used for biodiesel production can also be incorporated into industrial wastewater treatment to minimise production costs (Y. Wang *et al.*, 2016). Furthermore, vital factors such as the supply of light and carbon dioxide can significantly influence the efficiency of microalgae cultivation (Chew *et al.*, 2017). Challenges such as high installation and operating costs,

bad weather, and unstable source of illumination also can affect the microalgae industry (Yen *et al.*, 2013).

Thraustochytrids are osmoheterotrophic marine protists that can produce various types of fatty acids in the form of long-chained saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA). Commercially, thraustochytrids are known as fungi-like microalgae due to their heterotrophic mode of nutrition, which resembles that of fungi, and the absence of chloroplasts (Leyland *et al.*, 2017). They can grow in the absence of light and can release biflagellate zoospores for reproduction (S Raghukumar, 1996). Thraustochytrid strains with high SFA and MUFA content are preferable for lipid production and conversion into biodiesel. Biodiesel with a low degree of unsaturation will have a high cetane number, which can result in better fuel performance (Knothe *et al.*, 2003).

In contrast to photoautotrophic microalgae, thraustochytrids are cultivated heterotrophically by providing media rich with a carbon source such as glucose or from industrial by-products such as shochu distillery wastewater (Yamasaki *et al.*, 2006) and liquid residues from the beer industry (Quilodran *et al.*, 2009). Previous studies have demonstrated that culture media composition such as the type and concentration of carbon and nitrogen sources can significantly influence the biomass and lipid productivities of thraustochytrid (Leano et al., 2003). Several studies have assessed the potential of various strains of thraustochytrid in biodiesel production, yet the potential of tropical Malaysian thraustochytrid remains underexplored (Kim Jye Lee Chang *et al.*, 2012; Gupta *et al.*, 2016). The aim of the present study was to explore the optimal media composition for cultivation of Malaysian thraustochytrids with the potential for biofuel production. *Aurantiochytrium* sp. UMACC-T023, a thraustochytrid strain isolated from a mangrove area in Port Dickson, Negeri Sembilan, Malaysia, was demonstrated to have high SFA

and MUFA content of 77.2 ± 10.2 % total fatty acid (TFA) and 11.5 ± 1.7 % TFA, respectively (Ou *et al.*, 2016). High amount of SFA and MUFA may contribute to high total lipid content of this strain and later produce biodiesel which are good in oxidation stability (high SFA) and acceptable cold flow properties (high MUFA). These properties make this tropical thraustochytrid strain an ideal candidate for high production of lipid for biodiesel. UMACC-T023 appeared as orange-coloured colonies on a glucose-yeast extract-polypeptone (GYP) agar plate. This appearance is attributable to the ability of UMACC-T023 to produce carotenoids (Ou et al., 2016). Certain strains of thraustochytrids can produce carotenoids such as astaxanthin, β-carotene, and canthaxanthin (Yokoyama & Honda, 2007).

In previous studies, the optimisation study using response surface methodology (RSM) were only focusing on the production of docosahexaenoic acid (DHA) and the information regarding the optimisation of thraustochytrid strain for biodiesel production was scarce. In this dissertation, the media composition of UMACC-T023 was optimised for high biomass and lipid production using RSM with a central composite design (CCD). In comparison to one-factor-at-a-time (OFAAT) experimental design, RSM allows a substantial amount of information to be generated from a small run of experiments (Bezerra *et al.*, 2008). The effects of interactions between different variables on the response can also be evaluated using RSM (Bezerra et al., 2008). The variables prioritised for optimisation were glucose concentration, yeast extract concentration, and polypeptone concentration. The RSM model was validated in 1.4 L stirred-tank bioreactors for the evaluation of potential large-scale biodiesel production using thraustochytrid. The fatty acid composition of UMACC-T023 was also evaluated to assess whether it fulfilled the international standards for biodiesel.

## 1.2 Research objectives

The specific objectives of the present study are as follows:

- To enhance the biomass and lipid production of UMACC-T023 by optimizing glucose, yeast extract and polypeptone concentration using Response Surface Methodology (RSM) with Central Composite Design (CCD).
- To validate the optimum media composition in stirred-tank bioreactor for potential large-scale biodiesel production.
- To assess whether the biodiesel properties of Malaysian thraustochytrid strain UMACC-T023 fulfilled the international standards for biodiesel.

## 1.3 Problem statement

The biomass and lipid production of thraustochytrid strain is very much dependent on the use of culture medium. In order to enhance the biomass and lipid production of thraustochytrid, selected factors of the culture medium composition such as carbon source concentration and nitrogen source concentration need to be optimised and validated for large-scale production. No previous studies have focused on the use of RSM for biomass and lipid production of thraustochytrid for biodiesel application. Thraustochytrid strain UMACC-T023 has potential to be used as feedstock for biodiesel production. Thus, its biodiesel properties also need to be assessed to determine whether it fulfilled the international standards for biodiesel.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Thraustochytrids

Thraustochytrids are unicellular, osmoheterotrophic marine protists belonging to the kingdom Stramenopila (Humhal *et al.*, 2017). Thraustochytrids feature ectoplasmic nets produced from single or multiple bothrosomes (Porter, 1969). Thraustochytrids are known to be non-plastidial and have no chloroplast. Even though their ancestor was plastidial, it was believed that they lost their plastids at some point in the past (Leyland *et al.*, 2017). Thraustochytrids are heterotrophic microorganisms that obtain their nutrients by consuming organic matter such as mangrove leaves. Given that sodium is vital for their growth, thraustochytrids are exclusively marine, with diverse aquatic habitats. They can be found in tropical as well as polar regions, coral reefs, rocky coasts, coastal water, and deep sea (Leano, 2001; Liu *et al.*, 2014; Q. Wang *et al.*, 2019). The lipid content of thraustochytrids is varied with different strains but it can reach more than 50% of the dry weight under optimised conditions (Z. Y. Chi *et al.*, 2007; Jakobsen *et al.*, 2008; Liang *et al.*, 2010). Thraustochytrids can produce high amount of omega-3 fatty acids (more than 50% of TFA under optimised conditions), especially DHA (C22:6n3) and eicosapentaenoic acid (EPA) (Huang *et al.*, 2003; Quilodran *et al.*, 2009; Singh *et al.*, 1996). Thraustochytrids can produce PUFA through two different pathways, the fatty acid synthase (FAS) aerobic pathway and the polyketide synthase-like (PKS) anaerobic pathway (Marchan *et al.*, 2018). Normally, DHA is obtained from fish oil derived from species such as cod and salmon. DHA is an essential component of the cellular membranes of the visual and nervous system in humans and supports the development of these systems during early childhood (Simopoulos, 1989). Currently, DHA from thraustochytrid is commercially produced by Royal DSM (DHASCO®) and OmegaTech Inc. (DHAGold) (Ratledge, 2004).

## 2.2 Upstream processes

Upstream processes refer to all of the steps required in the fermentation process to cultivate and grow cells. In the following paragraphs, the main factors of upstream processing for thraustochytrid cultivation are explained. A visualised summary of the upstream processing of thraustochytrids is demonstrated in Figure 2.1.

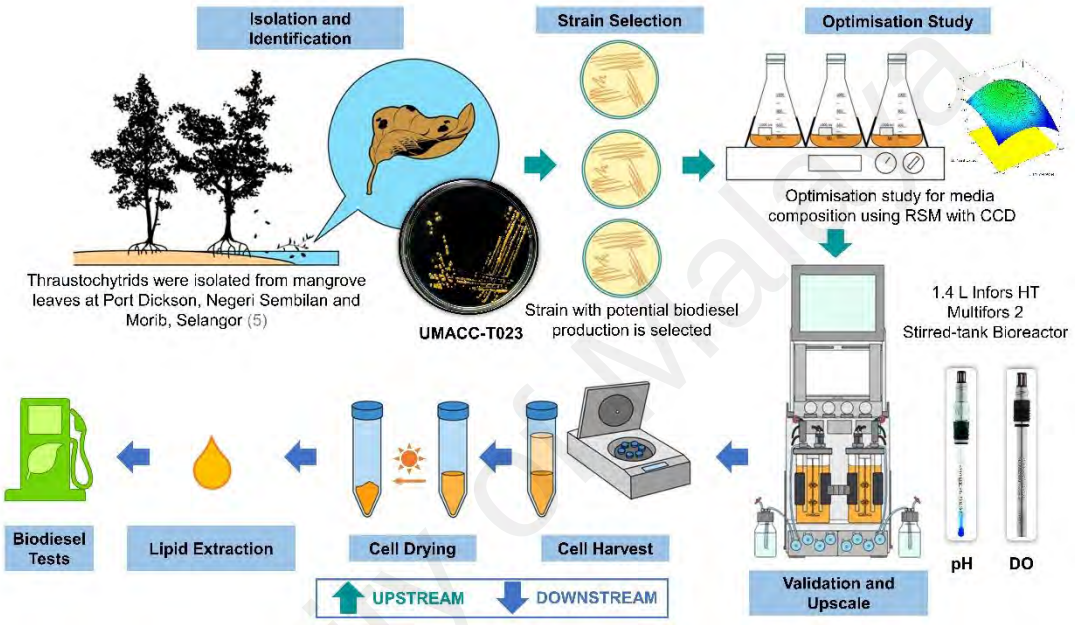


**Figure 2.1** Brief summary of the upstream and downstream processing of thraustochytrid for lipid and carotenoid productions. (Modified from Sohedein *et al.* (2020))

### 2.2.1 Strain selection

Achieving a high lipid production from thraustochytrids requires the selection of target species to optimise lipid production (Granata, 2017). When a specific strain has been chosen, the optimisation of lipid production can be performed. The optimisation process is conducted with the aim of increasing the cell growth rate, biomass, and lipid productivity of a specific strain (Granata, 2017). First, thraustochytrids are isolated from various habitats such as Antarctic water, cold and temperate waters, tropical waters, or

subarctic water. Next, among the many thraustochytrid strains being isolate, screening for a specific strain with a high production of biomass and targeted product such as DHA or carotenoids will be conducted. Researchers from different countries have put effort to isolates new strains of thraustochytrid and identify any potential strain for commercialisation. For example, in a study by Yang *et al.* (2010), 25 thraustochytrid strains were successfully isolated from marine habitats in Taiwan. Out of the 25 new strains, *Aurantiochytrium* strain BL10, with a high DHA profile, was selected for optimisation and lipid production. In another study, *Aurantiochytrium* sp. UMACC-T024 was chosen from 33 Malaysian thraustochytrids strains for an optimisation study due to its high biomass productivity (0.61 g $L^{-1}$ $day^{-1}$) and lipid productivity (70.86 mg $L^{-1}$ $day^{-1}$) (Ou *et al.*, 2016).

*Aurantiochytrium* sp. UMACC-T023 (UMACC-T023) is one of the 33 thraustochytrid strains which isolated from fallen leaves of Malaysian mangrove and maintained as uniculture at the University of Malaya Algae Culture Collection - Thraustochytrid (UMACC - T) (Ou *et al.*, 2016). All isolated strains in this study reached stationary phase on day 7, with specific growth rate ranged from 0.17 to 0.93 $day^{-1}$. This strain belongs to the genus *Aurantiochytrium*. In contrast with other thraustochytrid genus, the ectoplasmic nets of *Aurantiochytrium* strains are not well developed (Yokoyama & Honda, 2007). It has ovoid-shaped biflagellate zoospores. (Yokoyama & Honda, 2007) reported that *Aurantiochytrium* cells contain carotenoids such as astaxanthin, canthaxanthin, phoenicoxanthin and β- carotene. UMACC-T023 was found to produce high lipid and fatty acid contents (Ou *et al.*, 2016). Among the 33 Malaysian strains, UMACC-T023 produced the highest total fatty acid (TFA) with 85.9 mg $g^{-1}$ DW. 77.2% TFA of UMACC-T023 was saturated fatty acid (SFA), which make it a good candidate for biodiesel production. SFA such as palmitic acid and stearic acid both have high cetane number (CN) which make them a good source of biofuel. Higher CN means shorter

ignition delay (ID) time, the time that passes between injection of the fuel into the cylinder and onset of ignition and thus, provides higher fuel efficiency.

### 2.2.2 Optimisation study

One factor at a time (OFAAT) method are commonly used in optimisation study of thraustochytrids. However, when the optimisation study involves many factors, the number of runs for the experiment will be too large. Optimisation study using response surface methodology (RSM) will allow large amount of information to be generated from a small number of experiments (Bezerra *et al.*, 2008). In this study, the media composition of UMACC-T023 will be optimised for high biomass and lipid production by using response surface methodology (RSM) with central composite design (CCD). RSM also allow researchers to study and evaluate the interaction effect between various variables on the response. Analysis of variance (ANOVA) will be conducted to determine whether the model and variables are significant towards the response. Besides, 3D response surface profile can be generated and analysed to determine the optimum values.

### 2.2.3 Feeding strategy

The lipid content of thraustochytrids can vary depending on the medium composition and the growth conditions from 8% up to 81.7% of DW. In general, the maximum lipids production are found at the end of the exponential or the early stationary phase. (S. Raghukumar, 2008). Report shown that the thraustochytrids vary widely in their ability to use carbon and nitrogen sources and carbon to nitrogen (C:N) ratio play an important role for optimal biomass formation which then enhanced the lipid productivity (Yokochi *et al.*, 1998). For example, high carbon to nitrogen ratio (C:N) can contribute to higher lipid production of thraustochytrid (Ugalde *et al.*, 2018; Yokochi *et al.*, 1998). Therefore, thraustochytrids feeding strategy (mostly carbon and nitrogen source optimisation) is a crucial step for the production of DHA (Marchan *et al.*, 2018).

Different types of carbon sources have been studied for the cultivation of various strains of thraustochytrids. Similar strains fed with different carbon sources can give different results. The objective of varying the carbon source is to maximise biomass and lipid production from thraustochytrids as well as to reduce the cost of industrial-scale production. S. Raghukumar (2008) highlighted that, despite thraustochytrids from the same genus having similar lipid profiles, the total lipid content will vary depending on the strain, growth conditions, and composition of the medium being used (S. Raghukumar, 2008). According to a study by Chang *et al.* (2013), the highest biomass of 56.6 g $L^{-1}$ was achieved when pure glucose (lab-grade) was used to cultivate *Aurantiochytrium* sp. TC20 in a fed-batch fermentation as a carbon source, while the use of pure glycerol (lab-grade) achieved 55.9 g $L^{-1}$ of biomass. However, the DHA content when using pure glycerol (8.93 g $L^{-1}$) was higher than that when using pure glucose (8.23 g $L^{-1}$) (Chang *et al.*, 2013). These experiments show that different carbon sources used with the same strain can result in different amount of biomass and lipid productions. The high cost of using pure glucose, especially in industrial-scale production, is a significant disadvantage. Therefore, researchers have focused on finding low-cost carbon sources with similar or even better yields. In a study by Z. Y. Chi *et al.* (2007), crude glycerol, a primary by-product from the biodiesel industry, was used to cultivate *Schizochytrium limacinum* SR21. Although crude glycerol has low purity and a low economic value, the use of appropriate pre-addition treatment means that it can represent a cheaper alternative to pure glucose or glycerol. A biomass of 18.04 g $L^{-1}$ was obtained by Z. Y. Chi *et al.* (2007) when using crude glycerol compared with 18.47 g $L^{-1}$ from pure glucose. Interestingly, the use of crude glycerol as a carbon source demonstrated a higher specific cell growth rate (0.685 $day^{-1}$), maximum dry cell weight (18.04 g $L^{-1}$), and biomass productivity (3.06 g $L^{-1}$ $day^{-1}$) compared with pure glycerol.

Nitrogen is another media component essential for thraustochytrid growth and lipid production. Sources of nitrogen such as yeast extract and peptone have been extensively utilised by researchers in media for thraustochytrid cultivation. Other nitrogen sources which are commonly used include corn steep liquor (CSL), monosodium glutamate (MSG), and tryptone (Marchan *et al.*, 2018). Interestingly, the choice of nitrogen source can optimise the thraustochytrid production of a specific product. For example, Yokochi *et al.* (1998) optimised DHA production by *Schizochytrium limacinum* SR21 by using CSL as their nitrogen source. Their results showed higher total lipid content to biomass ratio when CSL was used as the nitrogen source compared with when yeast extract was used, likely because of the lower levels of nitrogen (5.5%) in CSL than in yeast extract (10%). Furthermore, the C:N ratio can significantly influence the total lipid content of thraustochytrid. For example, a high C:N ratio can promote the accumulation of TFA by thraustochytrids (Bowles *et al.*, 1999). The ratio of TFA content to dry cell weight in the *Schizochytrium limacinum* SR21 strain was two-fold higher when CSL was used compared with when yeast extract was used, indicating that this strain was able to accumulate a high total lipid content when CSL was used as the nitrogen source. The use of nitrogen depletion strategy for lipid accumulation is not something new. Other studies using microalgae treated with limited nitrogen sources demonstrated sharp increase in lipid content while decreasing in chlorophyll and proteins contents (Ho *et al.*, 2012; Sun *et al.*, 2014). These microalgae were speculated to utilise their chlorophyll and protein contents to convert their carbon structure into carbohydrate and lipid under the stress environment.

On the other hand, G. Q. Chen *et al.* (2010) demonstrated that complex nitrogen sources such as peptone, tryptone, and MSG were better choices than CSL to obtain high biomass by thraustochytrids. The biomass obtained when peptone, tryptone, and MSG being used were 8.81 g $L^{-1}$, 7.26 g $L^{-1}$, and 9.82 g $L^{-1}$, respectively. Meanwhile, the used

of CSL in various concentrations only resulted in biomass less than 4 g $L^{-1}$. Complex nitrogen sources provide an organic nitrogen source as well as other supplementary elements such as free amino acids, inorganic ions, fats, sugars, growth factors, and vitamins, which promote the growth of thraustochytrid (G. Q. Chen *et al.*, 2010).

### 2.2.4 Type of bioreactor

Increased agitation rate was found to reduce the biomass and DHA content of thraustochytrids (C. Y. Chen & Yang, 2018). Shear stress resulting from a high agitation rate can potentially influence biomass and DHA production. To further investigate this claim, C. Y. Chen and Yang (2018) studied the effects of mechanical and pneumatic agitation using stirred tank and air-lift bioreactors. However, these bioreactors have different designs, such as the use of different types of spargers, impellers, and baffles. Furthermore, the oxygen-transfer-rate (OTR) was kept constant in both bioreactors using a volumetric mass transfer coefficient (kLa) value of 27 $h^{-1}$. When the stirred tank bioreactor was used to cultivate *Thraustochytrium* sp. BM2, higher biomass productivity and DHA content were obtained compared with using air-lift bioreactor.

### 2.2.5 Agitation rate

The effects of aeration and agitation in a stirred-tank bioreactor on the growth and DHA production of thraustochytrids was studied by C. Y. Chen and Yang (2018). The studied strain, *Thraustochytrium* sp. BM2, was isolated from seawater in Beiman, Tainan, Taiwan and cultured in a 5 L stirred tank bioreactor. The results of this study indicated that in the absence of agitation (0 revolutions per minute (rpm)), the growth of *Thraustochytrium* sp. BM2 was significantly inhibited, while an increase in agitation rate increased the DO level in the culture broth and prevented oxygen depletion. The maximum biomass productivity of 4.1 g L-1 day-1 and DHA productivity of 266 mg L-1 day-1 were achieved at an agitation rate of 100 rpm. Interestingly, these results also

showed that there was a decrease in biomass and DHA content at a higher agitation rate (150 rpm). This reduction might be attributable to promotion of secondary metabolite production by hydrodynamic stress (Leckie *et al.*, 1991). The metabolic shift from DHA to secondary metabolite production may therefore have resulted in a lower DHA level. It is also important to note that the effect of agitation rate on DHA production may differ when a different bioreactor is used because of the effect of various other factors such as impeller type, sparger design, and baffles on the OTR. Therefore, to determine the rate of oxygen supply in the bioreactor, the volumetric mass transfer coefficient (kLa) can be calculated. A study by C. Y. Chen and Yang (2018) demonstrated a positive correlation between agitation rate and kLa, where higher agitation rates resulted in higher kLa values. The maximum biomass and DHA productivity achieved in this study was at an agitation rate of 100 rpm and kLa value of 23.4 ± 5.3 h−1.

### 2.2.6 Aeration rate

There are two well-known pathways for biosynthesis of PUFA, aerobic pathway and anaerobic pathway. The aerobic pathway uses desaturation and elongation procedure. The DHA biosynthesis process starts from stearic acid. On the other hand, anaerobic pathway utilise polyketide synthase (PKS). Anaerobic pathway catalyse the PUFA using the PUFA synthase (from polyketide synthase family) (Lippmeier *et al.*, 2009). The effect of different aeration rates on DHA production was studied by C. Y. Chen and Yang (2018) for aerobic fermentation of thraustochytrid. By controlling the agitation rate at 100 rpm, a low aeration rate was found to result in oxygen depletion within the first 20 hours of fermentation. However, higher aeration rates resulted in oxygen depletion only after 20 hours. These results demonstrated the correlation between dissolved oxygen (DO) and aeration rate in the fermentation medium. This study also demonstrated an increase in DHA content with increasing aeration rate. The highest DHA content of 8.65% TFA was achieved at an aeration rate of 1.2 vvm. A correlation was also observed between $k_{La}$ value

and aeration rate, with higher aeration rates resulting in higher $k_{La}$ values. These findings showed that a high aeration rate can increase DO in the media and result in improved DHA production by *Thraustochytrium* sp. BM2.

### 2.2.7 Impeller type and speed

Shear stress can be cause by the interaction between cells and the stirring apparatus of the bioreactor (C. Wang & Lan, 2018). In a stirred-tank bioreactor, impeller is used to provide mechanical agitation to the culture medium and improves the OTR. The choice of impeller type and speed can reduce the impact of shear stress towards thraustochytrid cells during fermentation. This is due to the high shear stress that can occur at the tip ends of the impeller (C. Wang & Lan, 2018). Different type of impeller being used can also influence the velocity of turbulent flow in the culture medium (C. Wang & Lan, 2018). The turbulent of flow is generated from the eddies and the energy content of the eddies is relative to its size. Shear stress can be reduced by choosing suitable impeller type and speed which reduce the size of the eddies being produced and thus has low turbulent flow (C. Wang & Lan, 2018). However, cell can also be damaged when the size of micro-eddies is equal or smaller than the cells (C. Wang & Lan, 2018). The smaller size micro-eddies can cause turbulent forces to the cells and cause shear stress which will damages the cells (C. Wang & Lan, 2018). The damage will be lessen when the micro-eddies have smaller dimension than the cells (C. Wang & Lan, 2018).

Impeller speed can be set at a higher rpm to improve the OTR in the fermentation medium. However, care should be taken when setting the upper limit of the impeller speed. Yaguchi *et al.* (1997) studied the effects of impeller type and speed on thraustochytrid growth in a bioreactor. They found that the growth rate increased when the agitation speed of a six-blade turbine impeller was increased from 100 rpm to 300 rpm. However, the growth rate was decreased when the agitation speed was set at 500

rpm. A similar effect was not found with the use of a propeller-shaped impeller, where the growth rate was not decreased even at 500 rpm. The research group assumed that oxygen transport was a limiting factor for thraustochytrid growth in a bioreactor, and that high impeller speed can improve oxygen transport and increase the growth rate. However, the turbine impeller can cause increased shear stress to cells compared with a propeller-type impeller at 500 rpm. Due to variation of shear stress with different impeller designs, the impeller speed should be optimised depending on impeller types and vessel size, and the shear stress on thraustochytrid cells should be observed during the optimisation study.

In contrast, Ganuza *et al.* (2008) were able to maintain a DO level above 30% by increasing the impeller speed from 300 rpm to a maximum of 700 rpm when using Rushton blade impellers in their bioreactor. Microscopic examination revealed that the thraustochytrids cells were not damaged by shear stress at the highest agitation rate (700 rpm). To summarise, the choice of impeller type and speed should be considered when designing or choosing a bioreactor for thraustochytrid cultivation (Figure 2.2). To prevent shear stress to thraustochytrid cells at high agitation rates, a Rushton blade impeller may represent a better option, followed by a propeller-type impeller. In Figure 2.3, a bioreactor design for the cultivation of thraustochytrid according to previous studies is presented. In this study, 1.4 L stirred-tank bioreactors equipped with Rushton blade impellers was used to prevent damage from shear stress towards thraustochytrid cells.

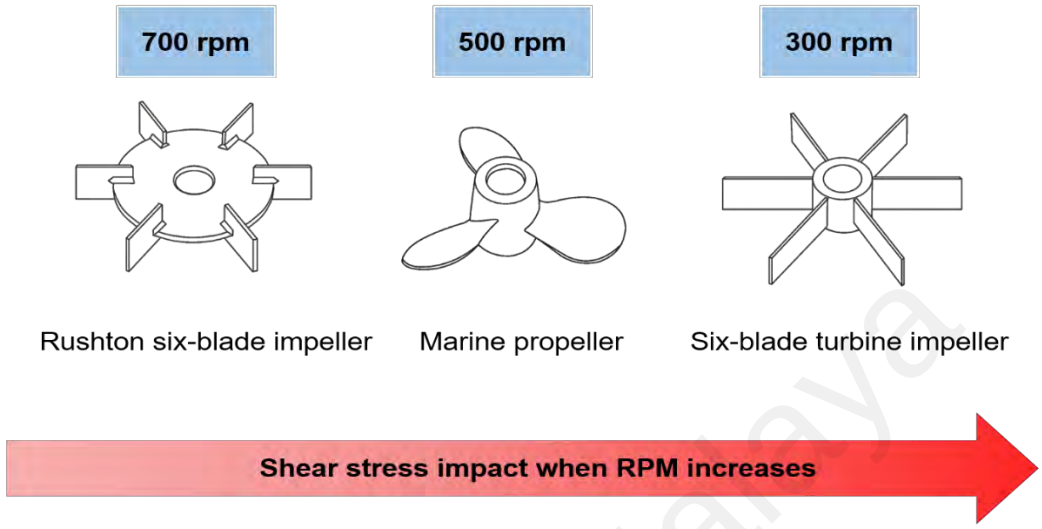


**Figure 2.2** Impeller type and RPM limit for the cultivation of thraustochytrid. (Adapted from Sohedein *et al.* (2020))

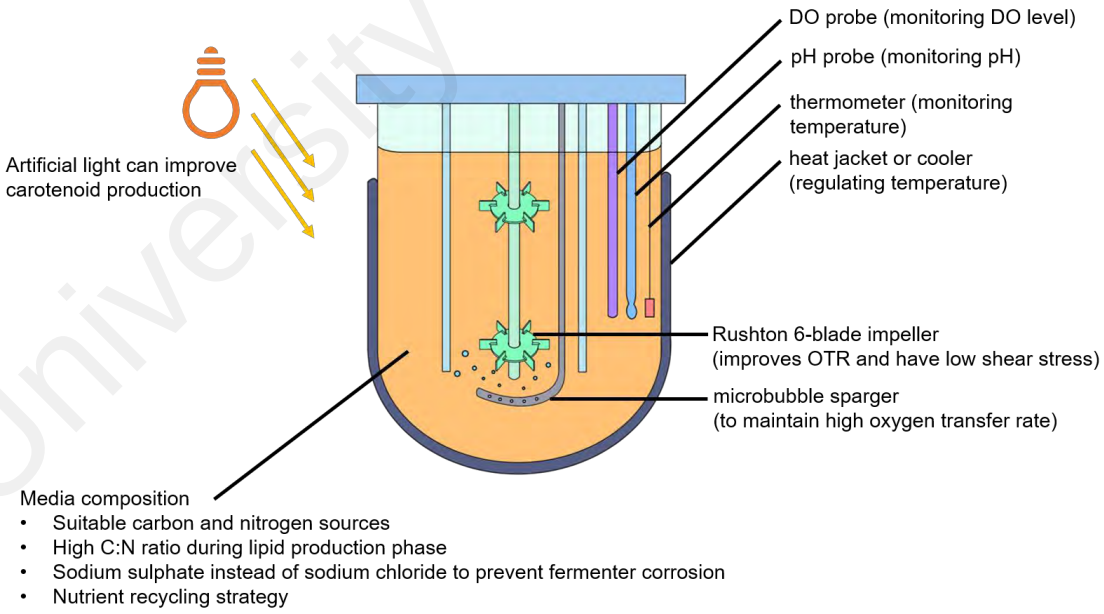


**Figure 2.3** Bioreactor design for the cultivation of thraustochytrid. (Adapted from Sohedein *et al.* (2020)).

### 2.2.8 Temperature

Temperature is a key factor that influences the biochemical process of lipid production in thraustochytrids. In a previous study, low temperatures were shown to limit cell growth but may increase the production of unsaturated fatty acids such as DHA (Taoka *et al.*, 2009). In another study in *Aurantiochytrium limacinum* sp. mh0186, a high percentage of DHA was obtained at 10°C, which decreased when the temperature was increased. A high unsaturated fatty acid content can improve membrane fluidity, leading to improved survivability in cold environments (de Mendoza & Cronan Jr, 1983). The gene expression of PUFA synthases in the polyketide synthase-like PUFA synthase (PKS) pathway in thraustochytrid has been studied by Ma *et al.* (2015). This study showed a significant increase in PUFA synthases in a cold stress setting. However, no significant expression was observed in type 1 fatty acid synthase involved in the fatty acid synthase (FAS) pathway for SFA production. This may explain the differences in fatty acid contents of thraustochytrids at low temperatures in comparison with high-temperature environments.

The cultivation of thraustochytrids in cold environments is preferable for PUFA production through the PKS pathway, while higher temperatures are better for biomass and SFA production through the FAS pathway (Ma *et al.*, 2015). The temperature of a bioreactor can be precisely controlled to create low- and high-temperature cultures. The biomass of thraustochytrids can be improved by setting the bioreactor at room temperature (25°C) during the logarithmic phase. At the stationary phase, in which lipid accumulation primarily occurs, the temperature of the bioreactor can be reduced to around 10–15°C. This two-phase temperature-controlled strategy may improve the production of PUFA such as DHA. The use of cold-shock treatment to improve the DHA production of thraustochytrid has also been demonstrated by Jain *et al.* (2004). However, this method seems to be strain-dependent. Taoka *et al.* (2011) studied the effect of cold-shock

treatment in *Aurantiochytrium limacinum* sp. mh0186 and found a significant increase in total lipid content after 72 h of cultivation at 10°C, although the percentage of unsaturated fatty acids such as DHA remained stable.

## 2.3 Downstream processes

Following the description of upstream processing above, a review of downstream processing is presented here. The key stages of the downstream process are as follows: cell harvesting, cell drying, lipid extraction, and conversion of lipids into biodiesel or secondary metabolites such as DHA and carotenoids. An effective cell disruption method is required to obtain better-quality lipids and higher product recovery while minimising the operating costs.

### 2.3.1 Cell disruption methods

The conventional lipid extraction processes require the dewatering and drying step for removal of water from the collected thraustochytrid biomass (Harris *et al.*, 2018). These steps could improve the efficiency of lipid extraction because the mass transfer of the lipids from the microalgae cells was not hindered by the residual water (C.-L. Chen *et al.*, 2015). However, the drying step is not economical due to high energy input needed (70% of total energy input for microalgae biodiesel production) (Sander & Murthy, 2010). Thus, the choice of cell disruption techniques that directly use the biomass slurry (wet route) instead of the dry biomass (dry route) can significantly reduce the total cost of biodiesel production from thraustochytrid. The purpose of cell disruption method is to release the intracellular lipids from thraustochytrid cells and allows the extracting solvent to react with the fatty acids. Some popular cell disruption techniques for microalgae biomass are classified into mechanical disruption methods such as the use of mortar and pestle, bead beaters, and high-pressure homogenizers (Halim *et al.*, 2012; Lee *et al.*, 2010; Montalescot *et al.*, 2015; D. Wang *et al.*, 2015). There is also pulse electric field (PEF)

method which utilise high voltage electric fields to create holes in cell membranes which release the cell contents (Joannes *et al.*, 2015). Six cell disruption methods were examined by Byreddy *et al.* (2017), including osmotic shock, grinding, bead vortexing, water bath immersion, sonication, and use of a shake mill to identify the most efficient strategy for breaking the cell walls thraustochytrids.

The osmotic shock method was found to be the most effective of the six methods examined followed by the grinding, sonication, and shake mill methods. The osmotic shock method requires a lower energy consumption in comparison with other methods and can be easily scaled-up. However, this method generates saltwater as a waste product and requires a longer operation time. The manual grinding method was found to be the quickest and most efficient method, although localised heating produced by the grinding mechanism may cause the denaturation of molecules.

The cell disruption methods for microalgae can also be studied and conducted on thraustochytrid cells. For example, methods such as high-pressure homogenization and sulfuric acid treatment demonstrated by Halim *et al.* (2012) showed effective disruption on microalgae cells.

### 2.3.2 Effect of organic solvents on efficiency of lipid extraction from thraustochytrids

Lipids from thraustochytrids are composed of various fatty acids with different polarities. The amount of extracted lipids relative to dry weight (DW) is determined by the suitability of the organic solvent used. Different solvents have different polarities, and the combination of various organic solvents can improve the total lipid content. The amount of lipid being extracted also influenced by the ratio of the solvents used.

In previous study, nine organic solvents and their combinations were examined by Byreddy *et al.* (2015). The solvents studied were chloroform, methanol, dichloromethane, diethyl ether, hexane, toluene, isopropanol, ethanol, and heptane. Lipid extraction using a single organic solvent showed that hexane produced the highest total lipid content (% DW) of 12.5%, followed by heptane (11%) and chloroform (9.7%). These three organic solvents were also tested in various combinations with other solvents, and the results showed that the combination of chloroform:methanol (2:1) was able to give the highest total lipid content of 22%. The higher lipid production resulting from the use of chloroform:methanol (2:1) indicated that thraustochytrid contains a higher proportion of polar and neutral lipids than of non-polar lipids. A mixture of non-polar and polar solvents can extract more lipids than by an individual organic solvent can. The combination of chloroform:methanol (2:1) can also extract carotenoids, hydrocarbons, triglycerides, phospholipids, sterols, and glycolipids.

However, the use of conventional solvent systems for lipid extraction demonstrate high total cost and not viable in large-scale production (Harris *et al.*, 2018). The organic solvents being used can also negatively impact the environment and cause health issues such as groundwater contamination, ozone depletion and carcinogenicity (Harris *et al.*, 2018). The application of alternative lipid extraction techniques such as the use of supercritical fluids and bio-based solvents can avoid these environmental and health problem (Sati *et al.*, 2019). A supercritical fluid is a substance that exist at a pressure and temperature above its critical point (Pourmortazavi & Hajimirsadeghi, 2007). Supercritical $CO_2$ is a popular supercritical fluid for the extraction of bio-compounds such as carotenoids and lipids and is recyclable (Goto *et al.*, 2015). In a study by Tang *et al.* (2011), they were able to achieve 33.9% of lipid yield and 27.5% of DHA content from *Schizochytrium limacinum* powder by using supercritical $CO_2$ at an optimized condition of 40 ℃, 35 MPa and ethanol (95% v/v) as the co-solvent. Bio-based solvents are green

solvents such as terpenes, ethyl lactate and methyl soyate that are mostly produced from agricultural sources (Sati *et al.*, 2019). For example, terpenes are derived from pine trees, tree leaf oils and citrus plants while ethyl lactate and methyl soyate can be obtained from citrus, corn and soybeans (Sati *et al.*, 2019). Interestingly, the use of bio-based solvent can not only improves the lipid yield from microalgae but also demonstrated low level of PUFA for a good biodiesel properties (Mahmood *et al.*, 2017).

### 2.3.3 Biodiesel production

Thraustochytrids resemble tiny factories of PUFA and MUFA production and can produce up to 90% MUFA (% TFA) such as eicosenoic acid, erucic acid, oleic acid, and palmitoleic acid (Arafiles *et al.*, 2011; Leano *et al.*, 2003). These fatty acids represent a promising feedstock for biodiesel production (K. J. L. Chang *et al.*, 2012). Marchan *et al.* (2017) reported that higher levels of unsaturated and short-chain saturated fatty acids (SC-SFA) produced by thraustochytrids improve the biodiesel quality and increase the oxidative rate and its thermal stability. Biodiesel rich with SC-SFA is desirable as it has good ignition properties, a low melting point, and a high cetane number, which indicates good biodiesel properties.

### 2.3.4 Biodiesel standards

Biodiesel converted from thraustochytrids needs to fulfil the quality requirements known as biodiesel standards before it can be used in blends or a substitute for automotive fuel. The standard specification for biofuel in the United States is termed ASTM D6751 while in Europe the biodiesel standard is known as EN 14214 (D. J. A. I. Astm, West Conshohocken, 2012). Among the specifications that need to be tested are density, viscosity, cloud point, cetane number, oxidation stability, and iodine value. Normally, thraustochytrid strains with high SFA and MUFA content are preferable for biodiesel production. Strains with a low degree of unsaturation are more likely to have an iodine

value and cetane number that meets the biodiesel standards. For example, *Schizochytrium* sp. PKU#Mn4 has a cetane number of 64.19, which meets the ASTM D6751 biodiesel standard minimum cetane number of 47 (Q. Wang *et al.*, 2018).

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 Strain selection and cultivation

The *Aurantiochytrium* sp. UMACC-T023 was acquired from the University of Malaya Algae Culture Collections and was subcultured in a glucose-yeast extract-polypeptone (GYP) agar containing glucose (10 g $L^{-1}$) (Bendosen Laboratory, Bendosen, Norway), yeast extract (2 g $L^{-1}$ ) (LabM, Heywood, UK), polypeptone (1 g $L^{-1}$ ) (LabM, Heywood, UK), 1.5% bacteriological agar (BD Diagnostic Systems, Maryland, USA), and artificial sea salt (30 g $L^{-1}$ ) (Instant Ocean, Sarrebourg, France) (Ou et al., 2016). The incubation conditions were 25°C ± 1°C, 120 rpm agitation, and absence of light. The fermentation procedure for the thraustochytrid rigidly followed the established fungal production blueprint of (Mohtar *et al.*, 2016; Wan-Mohtar *et al.*, 2019; Wan-Mohtar *et al.*, 2016).

## 3.2 Preparation of thraustochytrid inoculum

A loopful of UMACC-T023 colonies from a pure culture plate (Figure 3.1) was inoculated into 50 mL GYP liquid media in a 100 mL Erlenmeyer flask. The thraustochytrid culture was cultivated for three days in an incubator shaker (120 rpm) at 25 °C in the dark. Next, 50 mL of culture with an optical density of 1.0 at 620 nm ($OD_{620}$) was transferred to a 1 L Erlenmeyer flask containing 450 mL fresh GYP media at pH 7.5 (Fig. 1c). The optical density of the culture ($OD_{620}$) was measured using a UV-Vis spectrophotometer (Shimadzu UV 1800, Tokyo, Japan). The 1 L flask was then incubated in the incubator shaker under the same cultivation conditions until it reached $OD_{620}$ = 1.0 in the exponential phase (Ou et al., 2016).

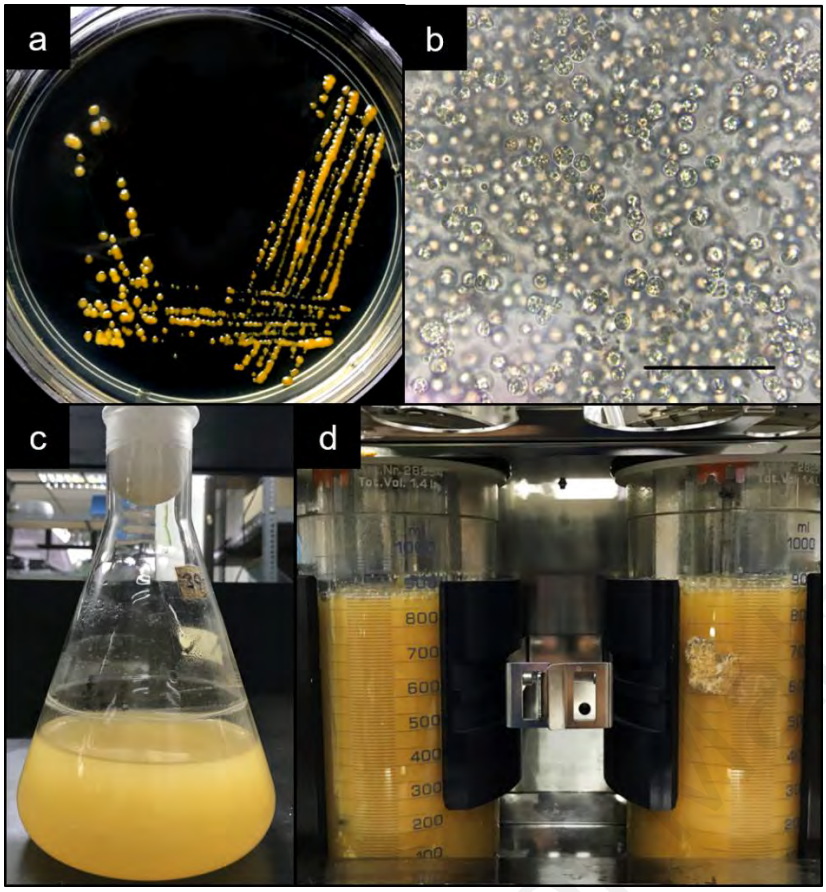


**Figure 3.1** (a) UMACC-T023 on GYP agar plate. (b) UMACC-T023 observed under light microscope (bar = 50µm). (c) UMACC-T023 in 450 mL GYP media broth. (d) UMACC-T023 in 1.4 L stirred-tank bioreactors. (Adapted from Sohedein *et al.* (2019)).

### 3.3 Optimisation of culture media using Response Surface Methodology

The optimisation of UMACC-T023 biomass and lipid production was conducted using batch fermentation in a 250-mL Erlenmeyer flask with 10% inoculum and working volume of 200-mL. The media composition of UMACC-T023, including glucose, yeast extract, and polypeptone, was optimised using RSM. CCD with the α-value set at 1.0 was selected. The experimental ranges and levels of the selected variables are presented in Table 3.1. The lowest minimum values for variables were glucose concentration, 10 g $L^{-1}$; yeast extract concentration, 2 g $L^{-1}$; and polypeptone concentration, 1 g $L^{-1}$; and the maximum values were glucose concentration, 80 g $L^{-1}$; yeast extract concentration, 20 g $L^{-1}$; and polypeptone concentration, 20 g $L^{-1}$. These ranges were selected and determined based on previous study (Ou *et al.*, 2016). The twenty experiments generated using CCD design in Design Expert 7.0 software are listed in Table 3.2.

The effect of variables and the interactions between them can be studied using an empirical model as shown in Eq 1. The empirical model followed a second-order quadratic model of the responses.

$$Y = b_0' + \Sigma_{i=1}^{n} b_i X_i + \Sigma_{i=1}^{n} b_{ii} X_i^2 + \Sigma_{i=1}^{n} \Sigma_{j>1}^{n} b_{ij} X_i X_j \qquad \textbf{Eq 1}$$

From Eq 1, $Y$ is the predicted response, $b_0'$ is the constant coefficient, $b_i$ is the linear coefficient, $b_{ii}$ is the quadratic coefficient, $b_{ij}$ is the interaction coefficient, and $X_i X_j$ are the coded values.

**Table 3.1** The selected variables, range and levels inputted for optimisation study. Reproduced from Sohedein *et al.* (2019).

| Variables | Range and levels | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| Glucose (g $L^{-1}$) | 10.0 | 45.0 | 80.0 |
| Yeast extract (g $L^{-1}$) | 2.0 | 11.0 | 20.0 |
| Polypeptone (g $L^{-1}$) | 1.0 | 10.5 | 20.0 |

**Table 3.2** CCD design with selected variables and actual responses for the biomass (DW) and total lipid content (% DW) of UMACC-T023. The experiments for the actual responses were performed in shake flasks (250 mL) under controlled conditions. Reproduced from Sohedein *et al.* (2019).

| Run No. | Variables | | | Actual Responses | |
|---|---|---|---|---|---|
| | Glucose (g $L^{-1}$) | Yeast extract (g $L^{-1}$) | Polypeptone (g $L^{-1}$) | Biomass (DW g $L^{-1}$) | Total lipid (% DW) |
| 1 | 80 | 20 | 1 | 3.50 | 10.20 |
| 2 | 45 | 11 | 10.5 | 3.85 | 11.95 |
| 3 | 80 | 11 | 10.5 | 3.10 | 20.02 |
| 4 | 10 | 2 | 20 | 2.71 | 17.81 |
| 5 | 45 | 11 | 10.5 | 3.51 | 7.12 |

**Table 3.2:** Continued.

| | | | | | |
|---|---|---|---|---|---|
| 6 | 10 | 20 | 1 | 3.12 | 12.71 |
| 7 | 45 | 11 | 20 | 3.83 | 7.48 |
| 8 | 10 | 20 | 20 | 4.83 | 4.97 |
| 9 | 45 | 2 | 10.5 | 2.46 | 11.13 |
| 10 | 80 | 20 | 20 | 3.83 | 8.62 |
| 11 | 45 | 11 | 10.5 | 3.48 | 12.36 |
| 12 | 45 | 11 | 10.5 | 3.92 | 11.73 |
| 13 | 45 | 11 | 10.5 | 3.22 | 15.22 |
| 14 | 10 | 11 | 10.5 | 3.23 | 19.50 |
| 15 | 80 | 2 | 20 | 3.46 | 10.12 |
| 16 | 10 | 2 | 1 | 2.64 | 16.90 |
| 17 | 80 | 2 | 1 | 3.06 | 10.68 |
| 18 | 45 | 20 | 10.5 | 3.72 | 7.89 |
| 19 | 45 | 11 | 10.5 | 3.20 | 9.38 |
| 20 | 45 | 11 | 1 | 2.99 | 8.47 |

## 3.4 Validation of the optimisation model in 250-mL shake flasks and 1.4-L stirred-tank Multifors 2 bioreactors

The RSM model was used to generate the optimised values for maximising biomass, lipid, and both biomass and lipid production of UMACC-T023. The optimised values were validated in 250-mL shake flask fermentation with 200 mL working volume and 10% inoculum. The validation experiment for both biomass and lipid production were also conducted in 1.4-L stirred-tank Multifors 2 bioreactors (INFORS-HT, Bottmingen, Switzerland; equipped with EVE Bioprocess Platform Software) for the assessment of potential large-scale thraustochytrid cultivation for biodiesel production (Fig. 1d). The bioreactors contained 10% inoculum, 750 mL GYP media maintained at 120 rpm agitation, 25$^{o}$C temperature, pH 7.5, and 1.0 vvm air input. The validation experiments

for both shake flasks and bioreactors were run for 7 days in the absence of light. Culture samples were collected during the stationary growth phase (day 7) for the measurement of biomass and total lipid content production. The stationary phase (day 7) was identified during preliminary study of growth phase of UMACC-T023 and also mentioned in previous study by Ou *et al.* (2016).

## 3.5 Analytical methods

### 3.5.1 Measurement of biomass

On day 7 of culturing, a 10-mL sample from each 250-mL flask was collected for biomass determination. An oven-dried and pre-weighed Whatman GF/C glass fibre filter was used to filter the sample. The filtered biomass was dried overnight in an 80 $^{o}$C oven, then maintained overnight in a desiccator and weighed (Ou et al., 2016).

### 3.5.2 Total lipid content determination

A 10-mL sample from the day 7 culture was collected and filtered through a Whatman GF/C glass fibre filter. A modified Bligh and Dyer (1959) method was used to extract the lipid from the thraustochytrid biomass. A 5 mL of methanol-chloroform (2:1 v/v) solvent was added to the filtered sample before hand-homogenised using a glass rod. The mixture was centrifuged at 3000 rpm for 10 minutes at 4 ℃. The supernatant was transferred to another 15 mL centrifuge tube by using a long glass Pasteur pipette. Then, 2 mL of chloroform and 2 mL of distilled water were added before the mixture was vortexed and centrifuged for 10 minutes (3000 rpm at 4 ℃). Then, the lower layer was transferred to oven-dried pre-weighed glass vials using long glass Pasteur pipette. The collected lipids were dried under nitrogen gas, maintained overnight in a desiccator, and then weighed. The total lipid content (% DW) was obtained by calculating the percentage of lipid relative to the sample biomass (DW).

### 3.5.3 Statistical analysis

The internal statistical tool in Design Expert 7.0 software (Stat-Ease, Minneapolis, USA) was used to perform analysis of variance (ANOVA) for the CCD quadratic model. Statistical significance for each of the model coefficients was indicated by values of $p < 0.05$.

### 3.5.4 Determination of biodiesel properties

The FAME of UMACC-T023 was analysed in various tests to determine its biodiesel properties and for assessment according to the international standards (D. ASTM, 2008). Kinematic viscosity was measured on Automatic Kinematic Viscosity Measuring System AKV-201 at 40 ℃ in accordance to ASTM D445. For carbon residue, the test was conducted using Micro Carbon Residue Tester ACR-M3 in accordance to ASTM D4530. The pour point and cloud point were determined using Mini Pour/Cloud Point Tester MPC-102 in a temperature range of -60 ℃ to 51℃. The pour point and cloud point were tested in accordance with ASTM D2500 and ASTM D6749, respectively. The cold filter plugging point was measured using Automated Cold Filter Plugging Point Tester AFP-102 in accordance to ASTM D637. 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 instrumentations provided by TANAKA Scientific Ltd., Tokyo. Besides, oxidation stability was also tested on Rancimat 743 (Methrom, Herisau, Switzerland) in accordance to EN 14112. For the measurement of ester content, monoglyceride, diglyceride, triglyceride, total glycerol content, water content, acid number, and iodine value, all the tests were conducted according to the European Standard Methods (CEN, 2019).

# CHAPTER 4: RESULTS AND DISCUSSION

## 4.1 Optimisation of UMACC-T023 for biomass production

The ANOVA results for biomass production of UMACC-T023 are presented in Table 4.1. It was found that 77.98% ($R^2$ = 0.7798) of the variability in the actual response could be described using this model. The *p*-value was 0.0219, indicating that the model was significant ($p < 0.05$). The regression model based on the actual factor of biomass can be expressed using Eq 2.

$$\textit{Biomass} = 1.99294 + 0.018902 \times \text{Glucose} + 0.10136 \times \text{Yeast Extract} - 0.011039 \times \text{Polypeptone} - 0.000710317 \times \text{Glucose} \times \text{Yeast Extract} - 0.000394737 \times \text{Glucose} \times \text{Polypeptone} + 0.00229532 \times \text{Yeast Extract} \times \text{Polypeptone} - 0.0000638219 \times \text{Glucose}^2 - 0.00189113 \times \text{Yeast Extract}^2 + 0.00184840 \times \text{Polypeptone}^2 \quad \textbf{Eq 2}$$

From the model, yeast extract (B, *p*-value = 0.0017) and polypeptone (C, *p*-value = 0.0124) showed significant effects on the yield of biomass at $p < 0.05$. However, negative effects were shown by glucose (A, *p*-value = 0.7109) and all quadratic terms (AB, AC, BC, $A^2$, $B^2$, and $C^2$). Figure 4.1 displays the merged effect of glucose, yeast extract, and polypeptone concentration in response surface profiles. Figure 4.1a shows the effect of glucose concentration (A) and yeast extract concentration (B), Figure 4.1b shows the effect of A and polypeptone concentration (C), and Figure 4.1c shows the effect of B and C on biomass production. By increasing the glucose concentration as shown in Fig. 4.1a and 4.1b, the sole carbon source provider only slightly increased biomass production. However, increasing yeast extract and polypeptone concentrations appeared to result in higher biomass production in comparison with glucose. Figure 4.1c shows the merged effect of both nitrogen sources, yeast extract and polypeptone, which resulted in high biomass production. The maximum biomass yield was obtained at a glucose

concentration of 10 g $L^{-1}$, yeast extract concentration of 20 g $L^{-1}$, and polypeptone concentration of 20 g $L^{-1}$. The results demonstrate that biomass yield was higher when the carbon source concentration was low and the nitrogen source concentration was high. This low carbon-to-nitrogen (C:N) ratio appears to improve biomass production, although previous studies have shown that a high C:N ratio can improve lipid production, as Bowles *et al.* (1999) and Jakobsen *et al.* (2008) demonstrated high lipid accumulation with media supplemented with a high C:N ratio. Nitrogen-starvation during fermentation of thraustochytrid was shown to increase the lipid production (Bowles *et al.*, 1999). These findings led to a multi-phase fermentation strategy in which a low C:N ratio was used in the initial phase of the fermentation followed by a high C:N ratio in the later phase to maximise lipid production (Rosa *et al.*, 2010).

**Table 4.1** Analysis of variance (ANOVA) results for the actual responses using CCD quadratic model for biomass production of UMACC-T023. Reproduced from Sohedein *et al.* (2019).

| Source | Sum of Squares | Mean Square | DF | *F* Value | Prob > *F* | |
|---|---|---|---|---|---|---|
| Model | 4.29 | 0.48 | 9 | 3.94 | 0.0219* | significant |
| A: Glucose | 0.018 | 0.018 | 1 | 0.15 | 0.7109 | |
| B: Yeast Extract | 2.18 | 2.18 | 1 | 17.99 | 0.0017* | significant |
| C: Polypeptone | 1.12 | 1.12 | 1 | 9.26 | 0.0124* | significant |
| AB | 0.4 | 0.4 | 1 | 3.3 | 0.0992 | |
| AC | 0.14 | 0.14 | 1 | 1.14 | 0.3114 | |
| BC | 0.31 | 0.31 | 1 | 2.54 | 0.142 | |
| $A^2$ | 0.017 | 0.017 | 1 | 0.14 | 0.7174 | |
| $B^2$ | 0.065 | 0.065 | 1 | 0.53 | 0.4824 | |
| $C^2$ | 0.077 | 0.077 | 1 | 0.63 | 0.4454 | |
| Residual | 1.21 | 0.12 | 10 | | | |

**Table 4.1:** Continued.

| | | | | | | |
|---|---|---|---|---|---|---|
| Pure Error | 0.46 | 0.092 | 5 | | | |
| Lack of Fit | 0.75 | 0.15 | 5 | 1.62 | 0.3042 | not significant |
| Cor Total | 5.51 | | 19 | | | |

| | | |
|---|---|---|
| Standard Deviation = 0.35 | Mean = 3.38 | Adequate Precision = 9.398 |
| $R^2$ = 0.7798 | Adjusted $R^2$ = 0.5817 | |

*Significant value

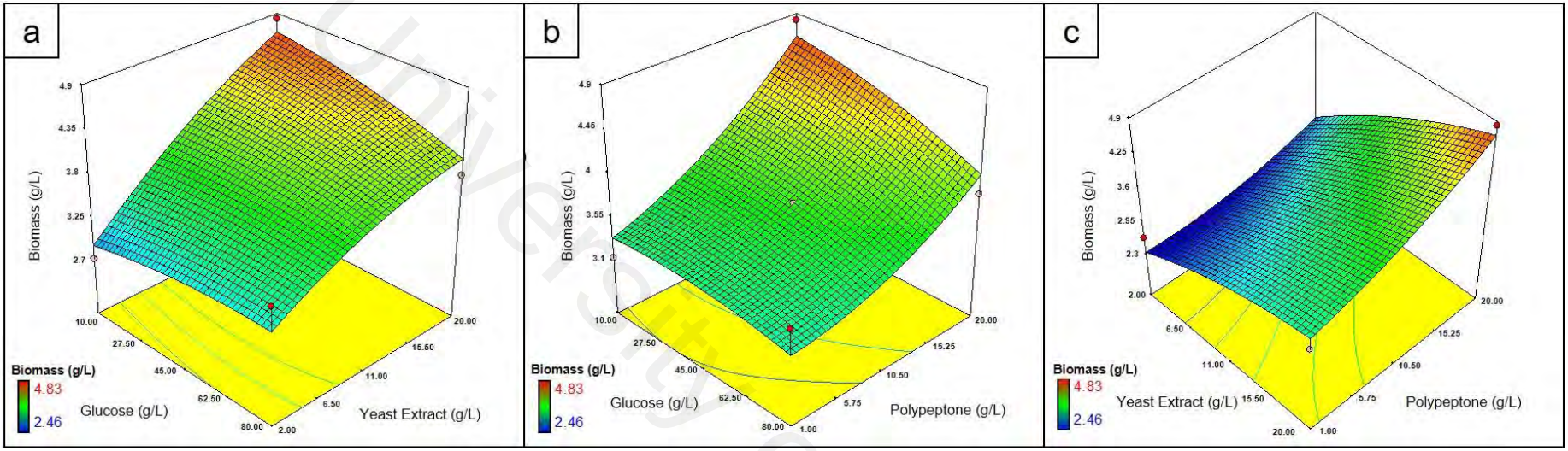


**Figure 4.1** Response surface profile of biomass production from UMACC-T023 displaying the effects between (a) Glucose and yeast extract, (b) Glucose and polypeptone, (c) Yeast extract and polypeptone. Reproduced from Sohedein *et al.* (2019).

## 4.2 Optimisation of UMACC-T023 for lipid production

ANOVA data for lipid production of UMACC-T023 are presented in Table 4.2. In total, 81.37% ($R^2$ = 0.8137) of the variability in the actual response can be described using this model. The *p*-value was 0.0068, which indicated that the model was significant ($p < 0.05$). The regression model based on the actual factor of lipid can be expressed using Eq 3.

$$\boldsymbol{Lipid} = 21.56737 - 0.65690 \times Glucose + 0.42864 \times Yeast\ Extract + 1.01059 \times Polypeptone + 0.00597222 \times Glucose \times Yeast\ Extract + 0.00176316 \times Glucose \times Polypeptone - 0.014137 \times Yeast\ Extract \times Polypeptone + 0.00597440 \times Glucose^2 - 0.036190 \times Yeast\ Extract^2 - 0.049489 \times Polypeptone^2 \quad \textbf{Eq 3}$$

From the model, yeast extract (B, *p*-value = 0.0146) was shown to have a significant effect on total lipid content (% DW) at $p < 0.05$. The quadratic term AB also showed a significant value, indicating a significant interaction between glucose and yeast extract concentrations on the lipid yield of UMACC-T023. Furthermore, the quadratic terms $A^2$ and $C^2$ also exhibited significant effects with $p = 0.0005$ and $p = 0.0112$, respectively. The standard deviation (SD) for the lipid results (SD = 2.39) was relatively higher compared with the SD in biomass results (SD = 0.35). This was because the maximum total lipid content (20.02 % DW) and the minimum total lipid content (4.97 % DW) obtained during the experiment run were comparatively higher than the maximum biomass (4.83 g $L^{-1}$ DW) and the minimum biomass (2.46 g $L^{-1}$ DW). Figure 4.2 shows the merged effect of glucose, yeast extract, and polypeptone concentration on lipid production displayed as response surface profiles. Figure 4.2a shows the effect of glucose concentration (A) and yeast extract concentration (B), Figure 4.2b shows the effect of A and polypeptone concentration (C), and Figure 4.2c shows the effect of B and C on total

lipid production. As shown in Figure 4.2a, a low concentration of glucose and yeast extract resulted in high total lipid production as indicated by the large red shaded area in the 3d plot. As shown in Figure 4.2b, all polypeptone concentrations were associated with a higher lipid production in comparison with various glucose concentrations. Figure 4.2c shows that both yeast extract and polypeptone showed a normal distribution for total lipid production at various concentrations. The maximum total lipid content production was obtained at a glucose concentration of 11.65 g $L^{-1}$, yeast extract concentration of 3.55 g $L^{-1}$, and polypeptone concentration of 10.77 g $L^{-1}$. The yeast extract concentration had a statistically significant effect on lipid yield ($p = 0.0146$), but the concentration needed to produce a high total lipid content was smaller than the required glucose concentration. This result demonstrated that a high C:N ratio can influence the lipid yield of UMACC-T023.

**Table 4.2** Analysis of variance (ANOVA) results for the actual responses using CCD quadratic model for lipid production of UMACC-T023. Reproduced from Sohedein *et al.* (2019).

| Source | Sum of Squares | Mean Square | DF | *F* Value | Prob > *F* | |
|---|---|---|---|---|---|---|
| Model | 281.41 | 31.27 | 9 | 5.49 | 0.0068* | significant |
| A: Glucose | 15.01 | 15.01 | 1 | 2.64 | 0.1356 | |
| B: Yeast Extract | 49.51 | 49.51 | 1 | 8.69 | 0.0146* | significant |
| C: Polypeptone | 9.92 | 9.92 | 1 | 1.74 | 0.2163 | |
| AB | 28.31 | 28.31 | 1 | 4.97 | 0.0498* | significant |
| AC | 2.75 | 2.75 | 1 | 0.48 | 0.5029 | |
| BC | 11.69 | 11.69 | 1 | 2.05 | 0.1824 | |
| $A^2$ | 147.3 | 147.3 | 1 | 25.87 | 0.0005* | significant |
| $B^2$ | 23.63 | 23.63 | 1 | 4.15 | 0.069 | |
| $C^2$ | 54.86 | 54.86 | 1 | 9.63 | 0.0112* | significant |

**Table 4.2:** Continued.

| | | | | | | |
|---|---|---|---|---|---|---|
| Residual | 56.94 | 5.69 | 10 | | | |
| Pure Error | 38.26 | 7.65 | 5 | | | |
| Lack of Fit | 18.68 | 3.74 | 5 | 0.49 | 0.7749 | not significant |
| Cor Total | 338.35 | | 19 | | | |
| Standard Deviation = 2.39 | | Mean = 11.71 | | | Adequate Precision = 8.472 | |
| $R^2$ = 0.8317 | | Adjusted $R^2$ = 0.6803 | | | | |

* Significant value

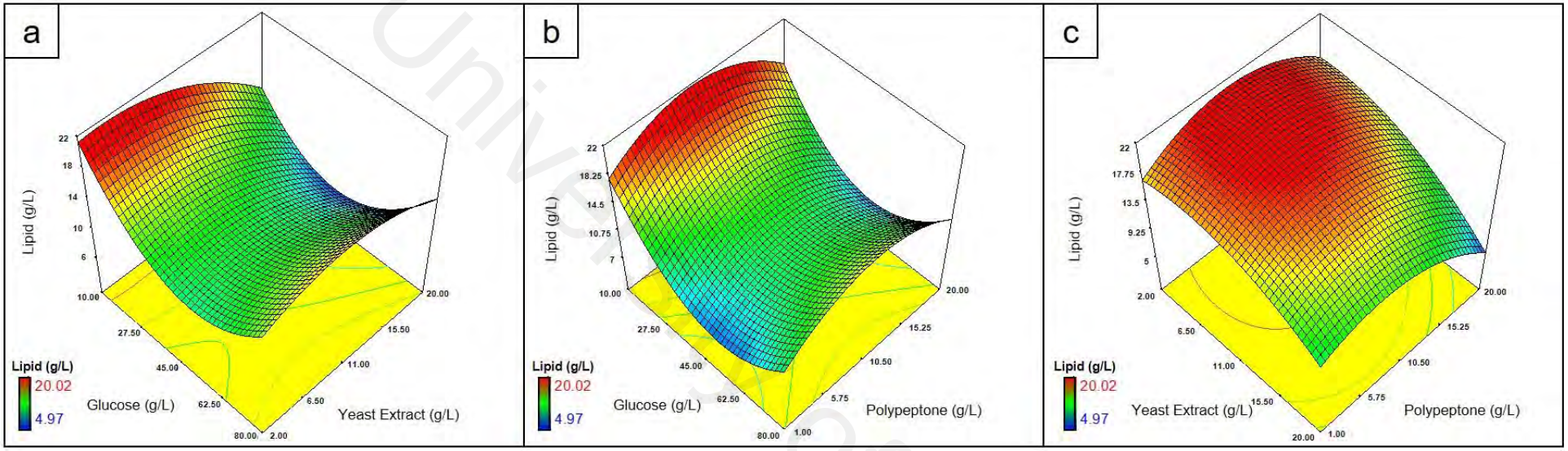


**Figure 4.2** Response surface profile of total lipid production from UMACC-T023 displaying the effects between (a) Glucose and yeast extract, (b) Glucose and polypeptone, (c) Yeast extract and polypeptone. Reproduced from Sohedein *et al.* (2019).

## 4.3 Validation of the optimised media composition

After construction of the biomass and total lipid CCD quadratic model, the biomass and total lipid production of UMACC-T023 was optimised using Design Expert software. Table 4.3 shows the composition of optimised media to validate the biomass and total lipid CCD quadratic model. The validation experiments were performed in 250-mL shake flasks and 1.4-L stirred-tank bioreactors under controlled conditions. Biomass production under optimised conditions was 4.83 g $L^{-1}$, while lipid production was 19.61 % DW. Interestingly, biomass production for UMACC-T023 in the stirred-tank bioreactor was 2.12-fold higher than in the shake flasks. However, total lipid production in the stirred-tank bioreactor was lower than that in the shake flasks. Fermentation conditions such as pH level, dissolved oxygen, and agitation speed can potentially influence the total lipid production from thraustochytrid in bioreactor. Stirred-tank bioreactors also have better aeration due to the use of impellers, which can aid the growth of UMACC-T023 and thus produce high biomass. Taken together, the validation results demonstrated that the model was valid for high biomass and lipid production of UMACC-T023.

**Table 4.3** Validation of the biomass and total lipid CCD quadratic model with the optimized media composition in 250 mL shake flasks and in 1.4 L stirred-tank bioreactors. Reproduced from Sohedein *et al.* (2019).

| Run | Variables | | | Predicted Response | | Actual Response | |
|---|---|---|---|---|---|---|---|
| | Glucose (g $L^{-1}$) | Yeast extract (g $L^{-1}$) | Polypeptone (g $L^{-1}$) | Biomass (g $L^{-1}$) | Total lipid (% DW) | Biomass (g $L^{-1}$) | Total lipid (% DW) |
| High biomass | 10 | 20 | 20 | 4.66 | - | 4.83 | 4.97 |
| High total lipid | 11.04 | 4.19 | 12.21 | - | 20.96 | 2.33 | 19.61 |

**Table 4.3:** Continued.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| High biomass and total lipid | 10 | 13.56 | 15.08 | 3.77 | 16.92 | 2.95 (SF)<br>6.25 (STB) | 19.32 (SF)<br>14.88 (STB) |

*SF = 250 mL shake flasks, STB = 1.4 L Stirred-tank bioreactors

## 4.4 Comparison with previous studies on thraustochytrid biomass and lipid optimisation

A comparison of optimisation methods for biomass and lipid production of thraustochytrid in previous studies is shown in Table 4.4. CCD and Plackett–Burman designs were frequently selected for the optimisation of thraustochytrid products such as biomass, docosahexaenoic acid (DHA), carotenoids, and squalene. To date, the fermentation working volume (750 mL) used for our *Aurantiochytrium* sp. UMACC-T023 was the highest compared to others at smaller volume which strongly suggested that the current study is fully equipped to be upscaled in a larger industrial Multifors-2 bioreactor for biodiesel production.

Even though other studies showed high biomass and lipid production, the thraustochytrid strains being optimised were for DHA production which is a disadvantage for biodiesel production due to high PUFA content. This study is the first to use RSM for optimisation of thraustochytrid growth specifically for biodiesel production. The amount of biomass and lipid produced by thraustochytrids appears to be strain-dependent, although the use of RSM resulted in higher production of biomass and lipid in all optimisation studies. The optimisation of lipid production can be improved by focusing on specific products such as PUFA for DHA or SFA and MUFA for biodiesel production.

Strain selection also plays an essential role in maximising the production of targeted fatty acids from thraustochytrids (N. T. L. Chi *et al.*, 2019).

**Table 4.4** Comparison of thraustochytrid optimisation study using response surface methodology (RSM) in literature. Reproduced from Sohedein *et al.* (2019).

| Optimization method | Thraustochytrid strain | Cultivation mode | Working volume (mL) | Product targeted | Maximum biomass ($g\ L^{-1}$) | Maximum total lipid (% DW) | Reference |
|---|---|---|---|---|---|---|---|
| CCD | *Aurantiochytrium* sp. UMACC-T023 | Industry Multifors 2 bioreactors | 750 | SFA, MUFA (biodiesel) | 6.25 | 14.88 | *This study* |
| CCD | *Schizochytrium* sp. SHG104 | Shake flask | 500 | PUFA (DHA) | 25.01 | 25.50 | Park *et al.* (2018) |
| CCD | *Aurantiochytrium* sp. SW1 | Shake flask | 50 | PUFA (DHA) | 17.80 | 53.90 | Manikan *et al.* (2015) |
| Plackett-Burman and CCD | *Thraustochytrium sp.* T01 | Shake flask | - | PUFA (DHA) | 27.01 | 13.50 | Chandrasekaran *et al.* (2018) |
| Plackett-Burman | Thraustochytrid F24-2 | Shake flask | 100 | PUFA (DHA) | 10.71 | 19.70 | Ugalde *et al.* (2018) |

## 4.5 Biodiesel properties of FAME converted from lipid produced by UMACC-T023.

The biodiesel properties of fatty acid methyl esters (FAME) from *Aurantiochytrium* sp. UMACC-T023 was tested and compared with the international standards for biodiesels (Table 4.5) (Ilham & Saka, 2012). Overall, the biodiesel properties of UMACC-T023 satisfied all requirements for international standard of biodiesel. The kinematic viscosity (4.8 $mm^2/s$), carbon residue (0.12%), ignition point (162.5 °C),

oxidation stability (5.8 h), ester content (97.1 %), total glycerol content (0.12 %), water content (287 mg/kg), acid number (0.24 mg(KOH)/g), and iodine value (108 g($I_2$)/100g) all fell within the range set by the EU (EN 14214) and US (ASTM D6751-08) standards.

**Table 4.5** Properties of fatty acid methyl esters (FAME) from *Aurantiochytrium* sp. UMACC-T023 for use as biodiesel in comparison with international standards[a]. Reproduced from Sohedein *et al.* (2019).

| Properties | Method | Unit | FAME (Biodiesel) | EU (EN 14214) | US (ASTM D6751-08) |
|---|---|---|---|---|---|
| Kinematic viscosity (40°C) | ASTM D445 | $mm^2/s$ | 4.8 | 3.5-5.0 | 1.9-6.0 |
| Carbon residue | ASTM D4530 | wt% | 0.12 | $\leq$ 0.30 | $\leq$ 0.05 |
| Pour point | ASTM D2500 | °C | 8 | - | - |
| Cold filter plugging point | ASTM D6371 | °C | - 6.4 | - | - |
| Ignition point | ASTM D93 | °C | 162.5 | $\geq$ 101 | $\geq$ 130 |
| Cloud point | ASTM D6749 | °C | -7.0 | - | - |
| Oxidation stability | EN 14112 | h | 5.8 | >6 | >3 |
| Ester content | EN 14103 | wt% | 97.1 | >96.5 | - |
| Monoglyceride | EN 14105 | wt% | 0.1 | <0.80 | - |
| Diglyceride | EN 14105 | wt% | 0.06 | <0.20 | - |
| Triglyceride | EN 14105 | wt% | n.d.[b] | <0.20 | - |
| Total glycerol content | EN 14105 | wt% | 0.12 | <0.25 | <0.24 |
| Water content | EN ISO12937 | mg/kg | 287 | <500 | <500 |
| Acid number | EN 14104 | mg(KOH)/g | 0.24 | <0.50 | <0.50 |
| Iodine value | EN 14111 | g($I_2$)/100g | 108 | <120 | - |

[a] CEN (2019); ASTM (2008) [b] n.d., not detectable.

# CHAPTER 5: CONCLUSION

Optimising the media composition of thraustochytrid UMACC-T023 resulted in a high biomass (6.25 g $L^{-1}$) and total lipid (14.88 % DW) with an optimised media composition of 10 g $L^{-1}$ glucose, 13.56 g$L^{-1}$ yeast extract, and 15.08 g $L^{-1}$ polypeptone. The yeast extract and polypeptone concentrations had a significant influence on biomass production while yeast extract concentration had a significant effect on the total lipid production of UMACC-T023. Biomass production also doubled when using a bioreactor compared with shake flask fermentation. The findings of this study will facilitate biomass and lipid production from other thraustochytrid strains for large-scale biodiesel production.

## REFERENCES


Amini, Z., Ong, H. C., Harrison, M. D., Kusumo, F., Mazaheri, H., & Ilham, Z. (2017). Biodiesel production by lipase-catalyzed transesterification of Ocimum basilicum L.(sweet basil) seed oil. *Energy conversion management, 132*, 82-90.

Arafiles, K. H. V., Alcantara, J. C. O., Cordero, P. R. F., Batoon, J. A. L., Galura, F. S., Leao, E. M., & Dedeles, G. R. (2011). Cultural Optimization of Thraustochytrids for Biomass and Fatty Acid Production. *Mycosphere, 2*(5), 521-531.

ASTM, D. (2008). 6571-08. Standard specification for biodiesel fuel blend stock (B100) for middle distillate fuels. *ASTM International*.

Astm, D. J. A. I., West Conshohocken. (2012). Standard specification for biodiesel fuel blend stock (B100) for middle distillate fuels.

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta, 76*(5), 965-977.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry Physiology, 37*(8), 911-917.

Bowles, R., Hunt, A., Bremer, G., Duchars, M., & Eaton, R. (1999). Long-chain n− 3 polyunsaturated fatty acid production by members of the marine protistan group the thraustochytrids: screening of isolates and optimisation of docosahexaenoic acid production. *J. Biotechnol., 70*(1-3), 193-202.

Byreddy, A. R., Gupta, A., Barrow, C. J., & Puri, M. (2015). Comparison of Cell Disruption Methods for Improving Lipid Extraction from Thraustochytrid Strains. *Marine Drugs, 13*(8), 5111-5127. doi:10.3390/md13085111

Byreddy, A. R., Rao, N. M., Barrow, C. J., & Puri, M. (2017). Evaluation of cell disruption method for lipase extraction from novel thraustochytrids. *Algal Research-Biomass Biofuels and Bioproducts, 25*, 62-67. doi:10.1016/j.algal.2017.04.025

Chandrasekaran, K., Roy, R. K., & Chadha, A. (2018). Docosahexaenoic acid production by a novel high yielding strain of Thraustochytrium sp. of Indian origin: Isolation and bioprocess optimization studies. *Algal Res., 32*, 93-100.

Chang, K. J. L., Dumsday, G., Nichols, P. D., Dunstan, G. A., Blackburn, S. I., & Koutoulis, A. (2013). High cell density cultivation of a novel Aurantiochytrium sp. strain TC 20 in a fed-batch system using glycerol to produce feedstock for biodiesel and omega-3 oils. *Applied Microbiology and Biotechnology, 97*(15), 6907-6918.

Chang, K. J. L., Dunstan, G. A., Abell, G. C., Clementson, L. A., Blackburn, S. I., Nichols, P. D., . . . biotechnology. (2012). Biodiscovery of new Australian thraustochytrids for production of biodiesel and long-chain omega-3 oils. *93*(5), 2215-2231.

Chang, K. J. L., Dunstan, G. A., Abell, G. C. J., Clementson, L. A., Blackburn, S. I., Nichols, P. D., & Koutoulis, A. (2012). Biodiscovery of new Australian thraustochytrids for production of biodiesel and long-chain omega-3 oils. *Applied Microbiology and Biotechnology, 93*(5), 2215-2231. doi:10.1007/s00253-011-3856-4

Chen, C.-L., Chang, J.-S., & Lee, D.-J. (2015). Dewatering and drying methods for microalgae. *Dry Technol, 33*(4), 443-454.

Chen, C. Y., & Yang, Y. T. (2018). Combining engineering strategies and fermentation technology to enhance docosahexaenoic acid (DHA) production from an indigenous Thraustochytrium sp BM2 strain. *Biochemical Engineering Journal, 133*, 179-185. doi:10.1016/j.bej.2018.02.010

Chen, G. Q., Fan, K. W., Lu, F. P., Li, Q. A., Aki, T., Chen, F., & Jiang, Y. (2010). Optimization of nitrogen source for enhanced production of squalene from thraustochytrid Aurantiochytrium sp. *New Biotechnology, 27*(4), 382-389. doi:10.1016/j.nbt.2010.04.005

Chew, K. W., Yap, J. Y., Show, P. L., Suan, N. H., Juan, J. C., Ling, T. C., . . . Chang, J.-S. (2017). Microalgae biorefinery: high value products perspectives. *Bioresour. Technol., 229*, 53-62.

Chi, N. T. L., Duc, P. A., Mathimani, T., & Pugazhendhi, A. (2019). Evaluating the potential of green alga Chlorella sp. for high biomass and lipid production in biodiesel viewpoint. *Biocatal Agric Biotechnol, 17*, 184-188.

Chi, Z. Y., Pyle, D., Wen, Z. Y., Frear, C., & Chen, S. L. (2007). A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. *Process Biochemistry, 42*(11), 1537-1545. doi:10.1016/j.procbio.2007.08.008

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances, 25*(3), 294-306. doi:10.1016/j.biotechadv.2007.02.001

Chu, S., & Majumdar, A. (2012). Opportunities and challenges for a sustainable energy future. *Nature, 488*(7411), 294-303. doi:10.1038/nature11475

de Mendoza, D., & Cronan Jr, J. E. (1983). Thermal regulation of membrane lipid fluidity in bacteria. *Trends in Biochemical Sciences, 8*(2), 49-52.

Ganuza, E., Anderson, A. J., & Ratledge, C. (2008). High-cell-density cultivation of Schizochytrium sp in an ammonium/pH-auxostat fed-batch system. *Biotechnology Letters, 30*(9), 1559-1564. doi:10.1007/s10529-008-9723-4

Goto, M., Kanda, H., & Machmudah, S. (2015). Extraction of carotenoids and lipids from algae by supercritical CO2 and subcritical dimethyl ether. *J. Supercrit. Fluids, 96*, 245-251.

Granata, T. (2017). Dependency of Microalgal Production on Biomass and the Relationship to Yield and Bioreactor Scale-up for Biofuels: a Statistical Analysis

of 60+Years of Algal Bioreactor Data. *Bioenergy Research, 10*(1), 267-287. doi:10.1007/s12155-016-9787-2

Gupta, A., Singh, D., Byreddy, A. R., Thyagarajan, T., Sonkar, S. P., Mathur, A. S., . . . Puri, M. (2016). Exploring omega-3 fatty acids, enzymes and biodiesel producing thraustochytrids from Australian and Indian marine biodiversity. *Biotechnology Journal, 11*(3), 345-355. doi:10.1002/biot.201500279

Halim, R., Harun, R., Danquah, M. K., & Webley, P. A. (2012). Microalgal cell disruption for biofuel development. *Applied Energy, 91*(1), 116-121.

Harris, J., Viner, K., Champagne, P., & Jessop, P. G. (2018). Advances in microalgal lipid extraction for biofuel production: a review. *Biofuel Bioprod Biorefin, 12*(6), 1118-1135.

Ho, S.-H., Chen, C.-Y., & Chang, J.-S. (2012). Effect of light intensity and nitrogen starvation on CO2 fixation and lipid/carbohydrate production of an indigenous microalga Scenedesmus obliquus CNW-N. *Bioresour Technol, 113*, 244-252.

Huang, J., Aki, T., Yokochi, T., Nakahara, T., Honda, D., Kawamoto, S., . . . Suzuki, O. (2003). Grouping newly isolated docosahexaenoic acid-producing thraustochytrids based on their polyunsaturated fatty acid profiles and comparative analysis of 18S rRNA genes. *Marine Biotechnology, 5*(5), 450-457.

Humhal, T., Kastanek, P., Jezkova, Z., Cadkova, A., Kohoutkova, J., & Branyik, T. (2017). Use of saline waste water from demineralization of cheese whey for cultivation of Schizochytrium limacinum PA-968 and Japonochytrium marinum AN-4. *Bioprocess and Biosystems Engineering, 40*(3), 395-402. doi:10.1007/s00449-016-1707-5

Ilham, Z., & Saka, S. (2012). Optimization of supercritical dimethyl carbonate method for biodiesel production. *Fuel, 97*, 670-677.

Jain, R., Raghukumar, S., & Chandramohan, D. (2004). Enhanced production of polyunsaturated fatty acid docosahexaenoic acid by thraustochytrid protists.

Jakobsen, A. N., Aasen, I. M., Josefsen, K. D., & Strom, A. R. (2008). Accumulation of docosahexaenoic acid-rich lipid in thraustochytrid Aurantiochytrium sp strain T66: effects of N and P starvation and O(2) limitation. *Appl Microbiol Biotechnol, 80*(2), 297-306. doi:10.1007/s00253-008-1537-8

Joannes, C., Sipaut, C. S., Dayou, J., Yasir, S. M., & Mansa, R. F. (2015). The potential of using pulsed electric field (PEF) technology as the cell disruption method to extract lipid from microalgae for biodiesel production. *Int J Renew Energy Res, 5*(2), 598-621.

Knothe, G., Matheaus, A. C., & Ryan III, T. W. (2003). Cetane numbers of branched and straight-chain fatty esters determined in an ignition quality tester☆. *Fuel, 82*(8), 971-975.

Leano, E. M. (2001). Straminipilous organisms from fallen mangrove leaves from Panay Island, Philippines. *Fungal Diversity, 6*, 75-81.

Leano, E. M., Gapasin, R. S. J., Polohan, B., & Vrijmoed, L. L. P. (2003). Growth and fatty acid production of thraustochytrids from Panay mangroves, Philippines. *Fungal Diversity, 12*, 111-122.

Leckie, F., Scragg, A. H., & Cliffe, K. C. (1991). Effect of Bioreactor Design and Agitator Speed on the Growth and Alkaloid Accumulation by Cultures of Catharanthus-Roseus. *Enzyme and Microbial Technology, 13*(4), 296-305. doi:Doi 10.1016/0141-0229(91)90147-3

Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., & Oh, H.-M. (2010). Comparison of several methods for effective lipid extraction from microalgae. *Bioresour Technol, 101*(1), S75-S77.

Leyland, B., Leu, S., & Boussiba, S. (2017). Are Thraustochytrids algae? *Fungal Biol, 121*(10), 835-840. doi:10.1016/j.funbio.2017.07.006

Liang, Y. N., Sarkany, N., Cui, Y., Yesuf, J., Trushenski, J., & Blackburn, J. W. (2010). Use of sweet sorghum juice for lipid production by Schizochytrium limacinum SR21. *Bioresource Technology, 101*(10), 3623-3627. doi:10.1016/j.biortech.2009.12.087

Lippmeier, J. C., Crawford, K. S., Owen, C. B., Rivas, A. A., Metz, J. G., & Apt, K. E. (2009). Characterization of both polyunsaturated fatty acid biosynthetic pathways in Schizochytrium sp. *Lipids, 44*(7), 621-630. doi:10.1007/s11745-009-3311-9

Liu, Y., Singh, P., Sun, Y., Luan, S. J., & Wang, G. Y. (2014). Culturable diversity and biochemical features of thraustochytrids from coastal waters of Southern China. *Applied Microbiology and Biotechnology, 98*(7), 3241-3255. doi:10.1007/s00253-013-5391-y

Ma, Z., Tan, Y., Cui, G., Feng, Y., Cui, Q., & Song, X. (2015). Transcriptome and gene expression analysis of DHA producer Aurantiochytrium under low temperature conditions. *Scientific Reports, 5*, 14446.

Mahmood, W. M. A. W., Theodoropoulos, C., & Gonzalez-Miquel, M. (2017). Enhanced microalgal lipid extraction using bio-based solvents for sustainable biofuel production. *Green Chem, 19*(23), 5723-5733.

Manikan, V., Kalil, M. S., & Hamid, A. A. (2015). Response surface optimization of culture medium for enhanced docosahexaenoic acid production by a Malaysian thraustochytrid. *Sci. Rep., 5*, 8. doi:10.1038/srep08611

Marchan, L. F., Chang, K. J. L., Nichols, P. D., Mitchell, W. J., Polglase, J. L., & Gutierrez, T. (2018). Taxonomy, ecology and biotechnological applications of thraustochytrids: A review. *Biotechnology Advances, 36*(1), 26-46. doi:10.1016/j.biotechadv.2017.09.003

Marchan, L. F., Chang, K. J. L., Nichols, P. D., Polglase, J. L., Mitchell, W. J., & Gutierrez, T. (2017). Screening of new British thraustochytrids isolates for

docosahexaenoic acid (DHA) production. *Journal of Applied Phycology, 29*(6), 2831-2843. doi:10.1007/s10811-017-1149-8

Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: A review. *Renew. Sust. Energ. Rev., 14*(1), 217-232. doi:10.1016/j.rser.2009.07.020

Mohtar, W. A. I. W., Ab Latif, N., Harvey, L. M., & McNeil, B. (2016). Production of exopolysaccharide by Ganoderma lucidum in a repeated-batch fermentation. *Biocatalysis and Agricultural Biotechnology, 6*, 91-101. doi:10.1016/j.bcab.2016.02.011

Montalescot, V., Rinaldi, T., Touchard, R., Jubeau, S., Frappart, M., Jaouen, P., . . . Marchal, L. (2015). Optimization of bead milling parameters for the cell disruption of microalgae: Process modeling and application to Porphyridium cruentum and Nannochloropsis oculata. *Bioresour Technol, 196*, 339-346.

Ou, M. C., Yeong, H. Y., Pang, K. L., & Phang, S. M. (2016). Fatty acid production of tropical thraustochytrids from Malaysian mangroves. *Botanica Marina, 59*(5), 321-338. doi:10.1515/bot-2016-0031

Park, H., Kwak, M., Seo, J., Ju, J., Heo, S., Park, S., & Hong, W. (2018). Enhanced production of carotenoids using a Thraustochytrid microalgal strain containing high levels of docosahexaenoic acid-rich oil. *Bioprocess Biosyst. Eng., 41*(9), 1355-1370. doi:10.1007/s00449-018-1963-7

Porter, D. (1969). Ultrastructure of Labyrinthula. *Protoplasma, 67*(1), 1-19.

Pourmortazavi, S. M., & Hajimirsadeghi, S. S. (2007). Supercritical fluid extraction in plant essential and volatile oil analysis. *J Chromatogr A, 1163*(1-2), 2-24.

Quilodran, B., Hinzpeter, I., Quiroz, A., & Shene, C. (2009). Evaluation of liquid residues from beer and potato processing for the production of docosahexaenoic acid (C22:6n-3, DHA) by native thraustochytrid strains. *World Journal of Microbiology & Biotechnology, 25*(12), 2121-2128. doi:10.1007/s11274-009-0115-2

Raghukumar, S. (1996). Morphology, taxonomy and ecology of thraustochytrids and labyrinthulids, the marine counterparts of zoosporic fungi.

Raghukumar, S. (2008). Thraustochytrid Marine Protists: Production of PUFAs and Other Emerging Technologies. *Marine Biotechnology, 10*(6), 631-640. doi:10.1007/s10126-008-9135-4

Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. *Biochimie, 86*(11), 807-815. doi:10.1016/j.biochi.2004.09.017

Rosa, S. M., Soria, M. A., Velez, C. G., & Galvagno, M. A. (2010). Improvement of a two-stage fermentation process for docosahexaenoic acid production by Aurantiochytrium limacinum SR21 applying statistical experimental designs and data analysis. *Bioresource Technology, 101*(7), 2367-2374. doi:10.1016/j.biortech.2009.11.056

Sander, K., & Murthy, G. S. (2010). Life cycle analysis of algae biodiesel. *Int J Life Cycle Assess, 15*(7), 704-714.

Sati, H., Mitra, M., Mishra, S., & Baredar, P. (2019). Microalgal lipid extraction strategies for biodiesel production: A review. *Algal Res., 38*, 101413.

Simopoulos, A. P. (1989). Summary of the NATO advanced research workshop on dietary omega-3 and omega-6 fatty-acids - biological effects and nutritional essentiality. *Journal of Nutrition, 119*(4), 521-528.

Singh, A., Wilson, S., & Ward, O. (1996). Docosahexaenoic acid (DHA) production by Thraustochytrium sp. ATCC 20892. *World J Microbiol Biotechnol, 12*(1), 76-81.

Sohedein, M. N. A., Wan-Mohtar, W. A. A. Q. I., Ilham, Z., Hui-Yin, Y., Supramani, S., & Siew Moi, P. (2019). Optimisation of Biomass and Lipid Production of a Tropical Fungal-like Microalgae Aurantiochytrium sp. UMACC-T023 in Submerged-Liquid Fermentation for Large-Scale Biodiesel Production. *Bioresour Technol*.

Sohedein, M. N. A., Wan, W. A. A. Q. I., Ilham, Z., Babadi, A. A., Hui-Yin, Y., & Siew-Moi, P. (2020). Vital parameters for biomass, lipid, and carotenoid production of thraustochytrids. *Journal of Applied Phycology*, 1-14.

Sun, X., Cao, Y., Xu, H., Liu, Y., Sun, J., Qiao, D., & Cao, Y. (2014). Effect of nitrogen-starvation, light intensity and iron on triacylglyceride/carbohydrate production and fatty acid profile of Neochloris oleoabundans HK-129 by a two-stage process. *Bioresour Technol, 155*, 204-212.

Tang, S., Qin, C., Wang, H., Li, S., & Tian, S. (2011). Study on supercritical extraction of lipids and enrichment of DHA from oil-rich microalgae. *J. Supercrit. Fluids., 57*(1), 44-49.

Taoka, Y., Nagano, N., Okita, Y., Izumida, H., Sugimoto, S., & Hayashi, M. (2009). Influences of culture temperature on the growth, lipid content and fatty acid composition of Aurantiochytrium sp. strain mh0186. *Marine Biotechnology, 11*(3), 368-374.

Taoka, Y., Nagano, N., Okita, Y., Izumida, H., Sugimoto, S., & Hayashi, M. (2011). Effects of cold shock treatment on total lipid content and fatty acid composition of Aurantiochytrium limacinum strain mh0186. *Journal of Oleo Science, 60*(5), 217-220.

Ugalde, V., Armenta, R. E., Kermanshahi-pour, A., Sun, Z. Y., Berryman, K. T., & Brooks, M. S. (2018). Improvement of culture conditions for cell biomass and fatty acid production by marine thraustochytrid F24-2. *J. Appl. Phycol., 30*(1), 329-339. doi:10.1007/s10811-017-1274-4

Wan-Mohtar, W. A. A. Q. I., Ab Kadir, S., Halim-Lim, S. A., Ilham, Z., Hajar-Azhari, S., & Saari, N. (2019). Vital parameters for high gamma-aminobutyric acid (GABA) production by an industrial soy sauce koji Aspergillus oryzae NSK in submerged-liquid fermentation. *Food Science and Biotechnology*, 1-11.

Wan-Mohtar, W. A. A. Q. I., Malek, R. A., Harvey, L. M., & McNeil, B. (2016). Exopolysaccharide production by Ganoderma lucidum immobilised on polyurethane foam in a repeated-batch fermentation. *Biocatal. Agric. Biotechnol., 8*, 24-31.

Wang, C., & Lan, C. Q. (2018). Effects of shear stress on microalgae–A review. *Biotechnol Adv, 36*(4), 986-1002.

Wang, D., Li, Y., Hu, X., Su, W., & Zhong, M. (2015). Combined enzymatic and mechanical cell disruption and lipid extraction of green alga Neochloris oleoabundans. *Int J Mol Sci, 16*(4), 7707-7722.

Wang, Q., Sen, B., Liu, X., He, Y., Xie, Y., & Wang, G. (2018). Enhanced saturated fatty acids accumulation in cultures of newly-isolated strains of Schizochytrium sp. and Thraustochytriidae sp. for large-scale biodiesel production. *Science of the Total Environment, 631*, 994-1004.

Wang, Q., Ye, H., Xie, Y., He, Y., Sen, B., & Wang, G. (2019). Culturable Diversity and Lipid Production Profile of Labyrinthulomycete Protists Isolated from Coastal Mangrove Habitats of China. *Marine Drugs, 17*(5), 268.

Wang, Y., Ho, S.-H., Cheng, C.-L., Guo, W.-Q., Nagarajan, D., Ren, N.-Q., . . . Chang, J.-S. (2016). Perspectives on the feasibility of using microalgae for industrial wastewater treatment. *Bioresour. Technol., 222*, 485-497.

Yaguchi, T., Tanaka, S., Yokochi, T., Nakahara, T., & Higashihara, T. (1997). Production of high yields of docosahexaenoic acid by Schizochytrium sp. strain SR21. *Journal of the American Oil Chemists Society, 74*(11), 1431-1434. doi:DOI 10.1007/s11746-997-0249-z

Yamasaki, T., Aki, T., Shinozaki, M., Taguchi, M., Kawamoto, S., & Ono, K. (2006). Utilization of shochu distillery wastewater for production of polyunsaturated fatty acids and xanthophylls using thraustochytrid. *Journal of Bioscience and Bioengineering, 102*(4), 323-327. doi:10.1263/jbb.102.323

Yang, H. L., Lu, C. K., Chen, S. F., Chen, Y. M., & Chen, Y. M. (2010). Isolation and Characterization of Taiwanese Heterotrophic Microalgae: Screening of Strains for Docosahexaenoic Acid (DHA) Production. *Marine Biotechnology, 12*(2), 173-185. doi:10.1007/s10126-009-9207-0

Yen, H.-W., Hu, I.-C., Chen, C.-Y., Ho, S.-H., Lee, D.-J., & Chang, J.-S. (2013). Microalgae-based biorefinery–from biofuels to natural products. *Bioresour. Technol., 135*, 166-174.

Yew, G. Y., Lee, S. Y., Show, P. L., Tao, Y., Law, C. L., Nguyen, T. T. C., & Chang, J.-S. (2019). Recent advances in algae biodiesel production: From upstream cultivation to downstream processing. *Bioresour. Technol. Rep.*, 100227.

Yokochi, T., Honda, D., Higashihara, T., & Nakahara, T. (1998). Optimization of docosahexaenoic acid production by Schizochytrium limacinum SR21. *Applied Microbiology and Biotechnology, 49*(1), 72-76.

Yokoyama, R., & Honda, D. (2007). Taxonomic rearrangement of the genus Schizochytrium sensu lato based on morphology, chemotaxonomic characteristics, and 18S rRNA gene phylogeny (Thraustochytriaceae, Labyrinthulomycetes): emendation for Schizochytrium and erection of Aurantiochytrium and Oblongichytrium gen. nov. *Mycoscience, 48*(4), 199-211. doi:10.1007/s10267-006-0362-0

## LIST OF PUBLICATIONS AND PAPERS PRESENTED

### PUBLICATIONS:

1. **Sohedein, M.N.A.**, Wan-Mohtar, W.A.A.Q.I., Ilham, Z., Hui-Yin, Y., Babadi, A.A., Phang, S.M. (2019). Vital Parameters for Biomass, Lipid and Carotenoid Productions of Thraustochytrids. *Journal of Applied Phycology* **(Accepted: 14th October 2019)**
2. **Sohedein, M.N.A.**, Wan-Mohtar, W.A.A.Q.I., Ilham, Z., Hui-Yin, Y., Supramani, S., Phang, S.M. (2019). Optimisation of Biomass and Lipid Production of a Tropical Thraustochytrid *Aurantiochytrium* sp. UMACC-T023 in Submerged-Liquid Fermentation for Large-Scale Biodiesel Production. *Biocatalysis and Agricultural Biotechnology*. **(Accepted: 7th January 2020)**

### CONFERENCE AND SEMINAR PRESENTATIONS:

1. Optimisation of Biomass and Lipid Production of a Tropical Thraustochytrid *Aurantiochytrium* sp. UMACC-T023 in Submerged-Liquid Fermentation for Large-Scale Biodiesel Production. Paper presented at the International Conference of Beneficial Microbes, 30th August – 1st July 2018, Kuching, Sarawak, Malaysia.