Evaluation of suitability of novel *Schizochytrium.sp* strain for biodiesel and high value PUFAs Production

By

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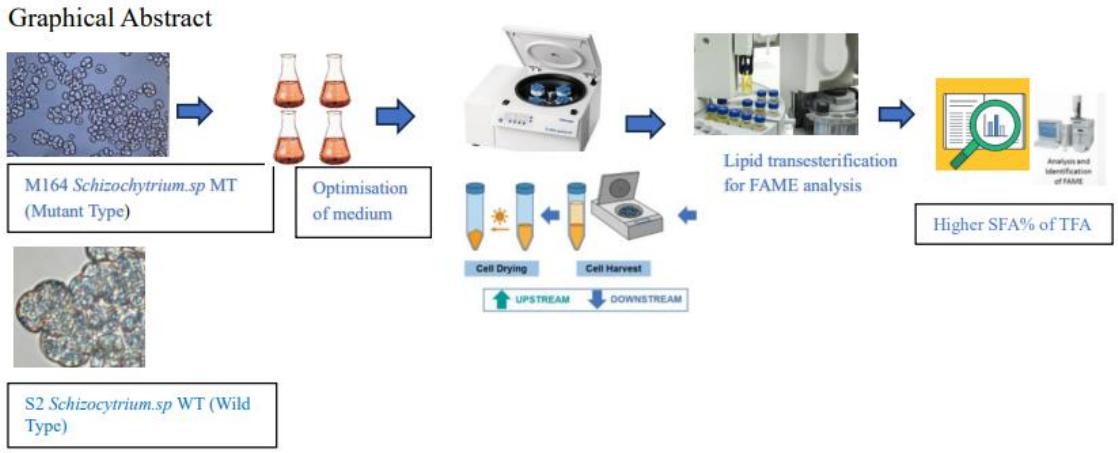
Thesis
Submitted to Flinders University
for the degree of masters of biotechnology

Master of Biotechnology
Flinders University
November 2023

Abstract

Marine oleaginous microbes are considered as a promising source for the sustainable production of biofuels such as biodiesel and high value polyunsaturated fatty acids, dietary fibre, minerals, and vitamins. These bio-actives have potential applications in different industries (i.e., nutraceuticals, food, animal feed and cosmetics). Biomass, oil and fatty acid yields dictate the suitability of these oleaginous microbes for commercialization. In this study growth conditions of a novel thraustochytrid strain were optimised using different carbon sources for improving biomass, lipid and fatty acid profile, to evaluate its suitability for biodiesel production. The potential of *Schizocytrium.sp* WT (wild type) and a *Schizochytrium.sp* isolate (M164) were tested for the extraction of lipids and various commercially high value products. Various carbon sources (glucose, glycerol, maltose and sucrose-) at different concentrations (3%, 5%, 7.5%, 10% and 12%) were tested. A maximum biomass of 27.3 g/L and lipid yield of 21.91% in M164 using glycerol as carbon source were obtained. While a maximum biomass of 23.92 g/L and lipid yield of 21.91% in glucose as carbon source were obtained. Different combination of solvents were conducted to assist in gaining higher saturated fatty acids (SFAs) and essential fatty acids by using different mixtures of polar and non-polar solvents. It was found that higher saturated fatty acid (SFA) up to 85% of the total fatty acids (TFA) was obtained from M164 *Schizochytrium.sp* isolate. This study supports that thraustochytrids could be employed for SFA extraction, the fatty acids suitable for biodiesel purposes.

Graphical Abstract



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Declaration

I declare that, to the best of my knowledge, this thesis does not contain any material that has been published or written by another person before, with the exception of instances where appropriate citations are made within the text. It also does not contain any material that has been approved for the granting of any degree or diploma. To the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Prakruthi Ravi Bhargav

November 2023

Acknowledgements

I would like to sincerely thank my supervisor Associate Professor Munish Puri and co-supervisor Dr. Mariam for being the inspiration behind my exploration of the intriguing field of single-cell oil extraction. In addition to shaping my present project, Mariam has genuine enthusiasm for the subject and her commitment to perfection have inspired me to pursue new goals. I've been inspired by her ability to overcome obstacles with grace and wisdom, and I hope to show myself the same amount of dedication in my own journey. Collaborating with her has not only improved my comprehension of the subject matter but also given me a feeling of direction and resolve. Her leadership approach, which embodies the ideal balance of direction and support, has established a benchmark for me to meet in my own work. This initiative is progressing because of her continuous commitment and forward-thinking mentoring. I am now deeply curious and committed to learning more about the nuances of this discipline because of her love for the subject. My comprehension and methodology have greatly benefited from her advice, and I sincerely appreciate the priceless insights provided by Dr. Mariam.

It has been a pleasure to work with them, and I value the tolerance and support they have shown on me during this process. Their dedication to quality and my capacity for motivation have improved the project and had a long-lasting effect on my development both personally and academically. I appreciate their dedication for creating a space that welcomes experimentation and creativity.

Additionally, I would like to thank Dr Adarsha Gupta and Shweta Sahni for their cooperation, as well as the biotechnology lab manager Kushari Burns for her ongoing assistance in the lab. A big thanks to my family and friends for supporting me. I would like to acknowledge Flinders university for smooth student experience.

List of Abberivations

FAME- Fatty Acid Methyl Ester

SFA- Saturated Fatty acid

MUFA- Mono-unsaturated Fatty Acid

PUFA- Polyunsaturated Fatty acid

IV- Iodine value

GC-FID – Gas chromatography flame ionization detector

CV- calorific value

SV- Saponification value

TAG- Triglycerides

DAGs- Diglycerides

MAGs- Monoglycerides

FFAs- Free fatty acids

DHA- Docosahexaenoic acid

EPA- Eicosapentaenoic acid

TFA- Total fatty acids

ALE- Adaptative laboratory Evolution

IEA- International Energy Agency

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Chapter 1

1.0.0 Introduction

The quest for alternative fuel sources continues to be a significant effort considering the growing worries over the rising fuel consumption. The depletion of fossil fuels, and the climbing greenhouse gas emissions has raised the global health concerns (Demirbas, 2009). The production of biofuels on a wide scale pose, particularly for the first- and second-generation biofuels, because of the unfavourable trade-offs, which brings global food security and food consumption issues (Acheampong et al., 2016). The hunt for an alternate source of sustainable fuel has been sparked by the challenges depletion of fossil fuels, rising prices, rising demand, and worries about global climate change (Abdullah et al., 2019). Fossil fuels have been a large topic of debate because of the release of CO_2 in the atmosphere which serves as a major contributor to global warming and climate change. Fossil fuels are finite resources that are becoming increasingly difficult and expensive to extract (Abdullah et al., 2019). According to few earlier reports, biofuels are predicted to supply up to 27% of the world's transportation fuel by 2050 (Subhash et al., 2022). Biodiesel is renewable, bio-degradable and has been associated with lesser carbon dioxide, carbon monoxide and hydrogen emission. It is regarded as the most promising sustainable alternative to all other current fuels (Alhattab and Brooks, 2020). It is necessary to identify sustainable substitutes that can supply the significant fuel volumes needed without jeopardising the use of agricultural land for food production. As a substitute feedstock for biodiesel, microalgae have gained interest. Globally, microalgae play a significant role in both CO_2 fixation and O_2 generation. They can absorb nutrients from effluents that would otherwise be released into the atmosphere and act as a buffer against rising CO_2 levels. As a result, the cultivation of microalgae significantly advances the fields of bioremediation and circular economy utilisation. As a result, marine microalgae has attracted attention as an alternative versatile feedstock (Bahadar & Khan, 2013). Furthermore, they do not require arable land for cultivation and have incredibly rich nutritional profile. They

are major sources of proteins, lipids, carbohydrates, polyunsaturated fatty acids (PUFAs), vitamins, and bioactive compound. Microalgae may be important components of alternative feedstocks to address the world's food shortage. Additionally, compared to plants, they collect a higher percentage of lipids in their biomass (Challagulla et al., 2015).Heterotrophic microalgae need expensive ingredients, mainly glucose as a carbon source, in order to produce biodiesel (Slade & Bauen, 2013). Glucose can make up to a third of the overall cost of fermentation production and as much as 80% of the medium cost (Li et al., 2007). Due to their diverse environmental adaptations, marine microbes have evolved to be highly metabolically efficient. In turn, they have evolved to use limited dissolved organic matter in a ratio where more metabolites have been produced than energy is consumed, allowing them to produce distinguished microbial metabolites (Dewapriya & Kim, 2014).

Fatty acid biosynthesis is species-specific and reliant on various cultivation methods. By adding various specialised abiotic variables, it is possible to increase the lipid content and the generation of certain fatty acids in culture, but high lipid content circumstances may not be most conducive to growth (Nautiyal et al., 2014). The most effective and well-known method for achieving high lipid accumulation in oleaginous microbes is, the combination of media optimisation, effective solvent extraction technology, and nitrogen starvation (Lee Chang et al., 2012). Sustainable low-cost carbon sources are necessary to lower these process costs and support the commercialisation of heterotrophic microalgae-derived biodiesel production. One strategy is to combine the development of microalgae with low-cost carbon sources, such as waste water rich in carbon or glycerol obtained from the biodiesel sector (Di Caprio et al., 2015, Quiroz Arita et al., 2015). Previous study has revealed that the solvent extraction efficiency of lipid in microalgae could be greatly improved if we address the importance of cell membrane and its permeability to various structures depending on the composition of the cell wall of the desirable productivity (Xiajie Ren et al.,2021).

Finding novel microalgae with higher lipid productivity can also reduce production costs (Ahmad et al., 2015). As previously stated, single-cell oil (SCO) is the term for the lipids produced by microorganisms and is a crucial feedstock for the companies that create biodiesel and omega-3 fatty acids. The subsequent steps in the manufacture of SCOs includes, culturing of the microorganisms, harvesting the biomass (the separation of the cells from the culturing media), the extraction of lipids, and finally, their purification.

1.1.0 Importance of Biofuels and Market

According to BCC Research (2023), the compound annual growth rate (CAGR) for the global nutraceutical and biodiesel secondary industry from 2021 to 2026 is expected to be 8.7%, or $289.8 billion to $438.9 billion for Omega-3. In the figure 2. he pie chart depicts the world feedstock for biofuel production. Due to their great consumer appeal, ingredients are in high demand. Sales of omega-3 components increased to US$ 7.4 billion in 2023 and during the anticipated period, at a 10.8% annual rate of growth of the omega-3 component. By 2033, the market is anticipated to bring in $20.6 billion (Gonçalves, 2023). The IEA's main analysis of the sector, Renewables 2021, is based on recent policy changes and commercial advancements. In addition to examining major industry difficulties and pointing out roadblocks to quicker expansion, it projects the use of renewable energy technology in heat, transportation, and power through 2026.Any energy transformation aimed at achieving net zero must start with renewables. A seamless transition to net zero depends on an awareness of the current role renewables play in the decarbonization of various industries as the world turns away from fossil fuels that create carbon emissions.

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Figure 1 (A&B) - Australian Government reports on world biofuels from 2022 December, The IEA's main analysis of the sector, Renewables 2021, is based on recent policy changes and commercial advancements. In addition to examining major industry difficulties and pointing out roadblocks to quicker expansion, it projects the use of renewable energy technology in heat, transportation, and power through 2026 (Gonçalves, 2023).

This is mostly because government around the world are changing their laws about renewable energies, and people are keen to find alternatives to fossil fuels. In Asia, for instance, Malaysia, a developing nation, has also actively promoted the growth of biodiesel usage by pledging to reduce the GHG emission intensity of the GDP by 45% by 2030 relative to its emissions in 2005. Japan, a developed nation, has committed to reducing its glasshouse gas emissions by 80% by 2050, and it is actively working to increase the use of biodiesel for the freight vehicles used by the transportation and automobile sectors. Biodiesel which refers to long chain alkyl fatty acid esters, is a sustainable and renewable fuel (Sadeghinezhad et al., 2014).

Significant worries about the ongoing rise in carbon emissions that fuel climate change have sparked a great deal of interest in microalgae-based goods and renewable fuels. However, scaling up and commercial development of algal technologies have been hampered thus far by sustainability and economic concerns associated with the production of naturally occurring algae. First generation biodiesel was produced from animal fats, waste oil, vegetable oil, and biomass by transesterification (Van Eijck et al., 2014).These effective biodiesel can be effective substitutes for typical diesel sources, which can reduce the overall quantity of pollution caused by the petroleum fuels used in car engines, given the rapid increase in the use of fossil fuels (Torres-Tiji et al., 2020). Following its preparation and purification, various ratios of Iranian-produced petroleum diesel are combined with the biodiesel. International standards are used to measure the physical properties of the mixes, including density, kinematic viscosity, cloud point, flash point, and pour point. These qualities are then compared to standard circumstances. Ultimately, the biodiesel/diesel best ratio is determined (Sadeghinezhad et al., 2014). According to Australian government, Department of Forestry and Fishery and the International Energy Agency (IEA), the demand for biofuel would rise by 27%–134% from 146 billion litres annually in 2020 to between 186 and 342 billion litres annually in 2026. This increase depends on the relative cost of biofuels in comparison to oil. Compared to the IEA, the OECD-FAO predicted a more moderate increase in biofuel usage (134 billion litres of biofuel in 2026).

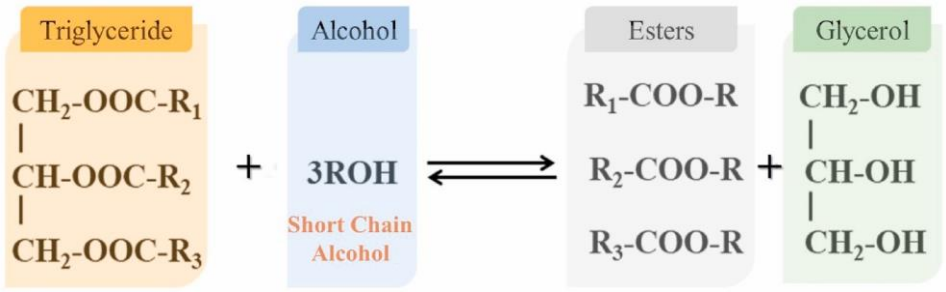

Figure 2- Representation of chemical reaction of triglyceride to short chain alcohol in the presence of acid that is termed as esterification. These triglyceride in the presence of base yields biodiesel with a byproduct glycerol. Biodiesel is fatty acid alkyl esters and is the product of the reaction. Depending on the comparison of the feedstock that includes various carbon sources will be influenced by these reactions to give biodiesel as a product.

1.2.0 Suitability of microalgal biodiesel

Although microalgae have been highlighted as a promising feedstock for biodiesel production, biodiesel from microalgal lipids needs to meet the required quality authorized by international standards such as European standards (EN 14214) and American Society for Testing and Materials (ASTM D6751) (de Jesus et al., 2020; Vignesh et al., 2020).

The fatty acids of microalgae are divided into saturated (without double bonds), monounsaturated (one double bond) and polyunsaturated (more than one double bonds) fatty acids with a 12–22 linear carbon length (Wu and Miao, 2014). The properties of biodiesel are highly contingent on the degree of saturation and a carbon length of fatty acid (Deshmukh et al., 2019). Saturated fatty acids provide storage stability to the biodiesel and protect it from autooxidation during a long-term storage, while unsaturated fatty acids with lower viscosity are beneficial in terms of cold flow characteristics (Wu and Miao, 2014).

The degree of unsaturation (DU), calorific value (CV), kinematic viscosity (KV), cetane number (CN), iodine value (IV), saponification value (SV), cold filter plugging point (CFPP), lubrication and oxidation stability are the primary parameters that determine the quality of microalgal biodiesel. The compositional matrix of fatty acid methyl esters (FAMEs) determines these absolute levels (Rinna et al., 2017). The fatty acids of microalgae are divided into saturated (without double bonds), monounsaturated (one double bond) and polyunsaturated more than one double bonds) fatty acids with a 12–22 linear carbon length (Wuand Miao, 2014). According to Deshmukh et al. (2019), the degree of saturation and carbon length of fatty acids have a significant impact on the characteristics of biodiesel. While unsaturated fatty acids with reduced viscosity are advantageous in terms of cold flow characteristics, saturated fatty acids give biodiesel storage durability and guard against autooxidation during long-term storage (Wuand Miao, 2014).

Improving the effectiveness of recovery and extraction techniques is essential to optimise the microalgal biodiesel production process. It is important to select chemical solvents carefully for the recovery and extraction process to maximise yields and reduce environmental effect. This will contribute to improving the production process for the overall efficiency of biodiesel. (K. Gerulová and colleagues, 2022). Using reported literature formulas (Sandani et al., 2020;

Srinuanpan et al., 2018; Valdez-Ojeda et al., 2015), a number of parameters, including cetane number, saponification value, iodine value, degree of unsaturation, long-chain saturation factor, and cold filter plugging point, were calculated to determine the biodiesel attributes of microalgal lipid. These computation offers important new information about the quality of biodiesel and whether it is suitable to use as a sustainable fuel. Furthermore, compliance with international standards like those set by the American Society for Testing and Materials and the European Union is crucial for microalgal biodiesel. These specifications guarantee that the biodiesel satisfies the necessary requirements for lubrication, ignition, and other properties. Research on alternative fuels has been prompted by the growing need for sustainable energy sources, with biodiesel emerging as a potential contender, *Schizochytrium.sp* Mutant 164 has lipid-rich makeup that offers a promising route for the synthesis of biodiesel.

1.3.0 Classification of biofuels

Figure 3-Representation of different classes of biofuels based on feedstock for the utilisation in production of biofuel such as first generation, second generation, third generation and fourth -generation. Image was taken from the book The sustainability biofuels 2022, source (Srivastava et al., 2019)

Biofuel is classified into first, second, and third generations based on the feedstock utilised for production (Abdullah et al., 2019). First generation of fuels are based on edible biomass as this encounter's global food needs, while second generation biofuels use non-edible biomass (agricultural by-products) which need fertile soil to thrive as limitations related to the cost-effectiveness involved in scaling up the production to commercial level and third generation of biofuel use the incorporation of microorganisms in the feedstock, while fourth generation biofuel focusses on modifying these microorganisms genetically. Unicellular marine protists known as thraustochytrids have been shown to collect significant amounts of lipids, making them a desirable feedstock for the generation of biodiesel (Gupta et al., 2016).

Biodiesel or fatty acid alkyl esters is the product of the reaction between a short chain alcohol and a long-chain free fatty acid or a triglyceride. Depending on the composition of the feedstock, biodiesel can be produced using the esterification reaction, or the two-step esterification and transesterification combined as shown in Figure 1.3

1.4.0 First-second generation fuels

The unintended consequence of using edible oils to produce first-generation biodiesel is that there is now more competition for food crops world wide (Piniz et al.,2014). Second generation biofuels utilize crops that are non-competing such as mahua,jatropha, karanja, jojoba as well as utilization of waste cooking oil, industrial waste, agricultural waste and animal fats. Meanwhile the use of inedible crops reduces the competition with edible food crops, second generation biofuels still compete with arable land, however impacts negatively for the world food supply and can lead to devastation of land resources, soil contamination (Kiss&Bildea,2012)

1.5.0 Third generation fuels

As we all know these feedstocks cannot meet the current increasing demand of energy (Nautiyal et al., 2014). The primary scenario predicts that between 2021 and 2026, the world's demand for biofuels will increase by 41 billion litres, or 28%. Half of this demand surge can be attributed to the demand recovering to pre-Covid-19 levels. The remaining increase is primarily driven by government policies, although additional factors influencing where development happens and which fuels expand fastest include costs, the overall demand for transportation fuels, and the design of specific policies based on the information provided by Australian government, Department of Forestry and Fishery and the International Energy Agency (IEA).

1.6.0 Fourth generation fuels

In addition to utilizing the lipids for biofuel production, microalgae are capable of producing other high value bioproducts, such as proteins, enzymes, polyunsaturated fatty acids (PUFA), carotenoid pigments, and exopolysaccharides (EPS). The formation of high-value coproducts during biofuel production is desirable when it adds greater value to the production process and improved process economics (Li et al. 2008; Stephens et al. 2010; Wijffels and Barbosa 2010).

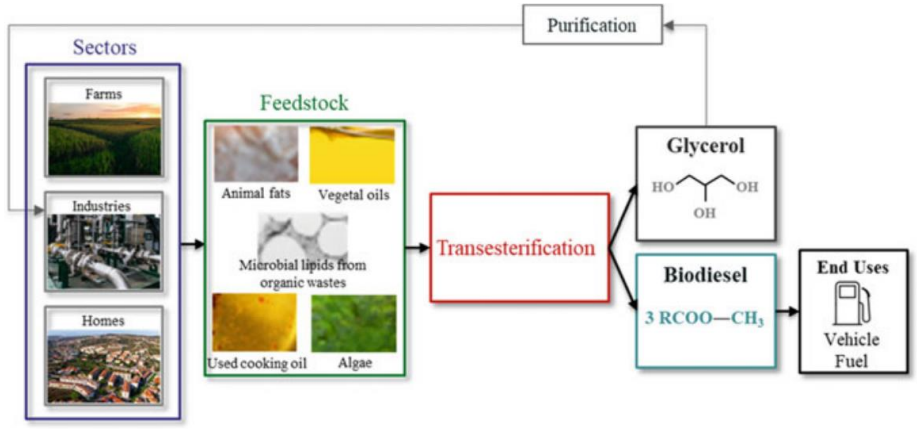

Figure 4 Representation of bicircular economy using different sectors like farm, industries, homes as mode of feedstock, these feedstocks might be animal fats, vegetable oil, microbial lipids from organic wastes, used cooking oil and different algae. By transesterification, we can produce biodiesel correspondingly end uses will be for vehicle fuel

Most of the world's energy needs have been satisfied by fossil fuels since the start of the industrial revolution. About 81% of the world's energy needs in 2017 came from petroleum (including heavy and light crude oils), coal, and natural gas—a proportion that has remained constant over the previous three decades (Newell et al., 2016).

1.8.0 Lipid extraction using solvents

To get the lipids accumulated in the microalgal cells as a result of various cultivation processes, lipid extraction is an essential step. These lipids may include enriched SCOs (SFAs/MUFAs) or functional foods (for human nutrition), as well as saturated fatty acids (SFAs, like palmitic acid and stearic acid), monounsaturated fatty acids (MUFAs, like oleic acid), and polyunsaturated fatty acids (PUFAs, like arachidonic acid, ARA; eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA), each with varying applications. a range of lipid extraction techniques, including solvent-based techniques, mechanically assisted techniques, and solvent-free techniques (Kariam et al., 2020). The method by Bligh and Dyer (1959) is typically the first to be cited when discussing a classic example of a lipid extraction technique, largely because of its simplicity and use of common solvents like methanol, chloroform, and water mixtures. The benefits of using solvents include low costs and the absence of pretreatment or mechanically assisted cell disruption.

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Figure 5 Lipid extraction using solvents, organic solvents, which include polar and non-polar solvents for obtaining higher lipid yield % and understanding the nature of the cell membrane (Karim et al.,2020)

Large amounts of lipids, such as the high-value omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), can be accumulated by a variety of marine microorganisms (Gupta et al., 2021).Oleaginous microbes are microorganisms that can collect more than 25% of their biomass as oil. The greatest producers of lipids include a variety of microorganisms, including fungi, yeast, microalgae, and thraustochytrids.

Summary of various methods employed used for lipid extraction

Methods	**Advantages**	**Limitations**	**References**
Bead milling	Rapid disruption of cells; feasible technique for mechanically disrupting cells on a massive scale	The degree of disruption is dependent on the properties of the bead; in large-scale applications, a significant amount of energy is needed.	(Joannes et al., 2015)
High-speed homogenisation	Quick extraction time; high rate of cell disruption; excellent effectiveness	High energy usage, unsuitable for widespread use	(Günerken et al., 2015)
High-pressure homogenisation	Effective, quick cell disruption that is	quick disruption, but in general a lesser yield of	(Show et al., 2015)

	appropriate for scaling up	lipids than with other techniques	
Ultra-sonication	Reduced extraction time, increased solvent penetration into cellular components, decreased solvent consumption, and enhanced intracellular content release	A significant amount of cell debris is discharged, making the separation procedure more difficult.	(Halim et al., 2013)
Microwave assisted	Reduced extraction time, increased solvent penetration into cellular components, decreased solvent consumption, and enhanced intracellular content release	Excessive power usage and challenging to expand	(Y. Zhang et al., 2022)
Pulse electric field	Relatively safe , simple and economical in lab scale Quick disruption, minimal energy usage, and no chemical addition	Large-scale maintenance is a limiting factor that can lead to the creation of free radicals	(Zheng et al., 2011)
Hydrothermal liquification	Water is used during the extraction process to produce high-quality, environmentally friendly biocrude.	High energy consumption because of the extremely high temperature involved; further research is required	(J. Zhang et al., 2022)

Organic solvent	High oil yield, very efficient, and reasonably priced	Solvents are extremely poisonous and/or combustible; recovering them requires a lot of money and energy, and a lot of solvent is needed.	(Kumar et al., 2017)
Ionic liquid	High oil yield, reusable, and short extraction time	Certain ionic liquids are exceedingly costly and hazardous to the environment.	(Kumar et al., 2017)
Nanoparticle	High reusability, low energy consumption, and efficiency	Some are highly costly; for commercial applications, the cost of synthesis needs to be assessed.	(Huang et al., 2014)
Oxidation	An effective method; high yield, high saturated More works need to be done	hydrocarbon goods for widespread usage Easy extraction with minimal energy	(Faried et al., 2017)
Osmotic shock	Easy extraction with minimal energy usage	Production of waste salt water, which takes time	(Kadir et al., 2018)
Super critical fluid	High oil yield, non-flammable, and non-toxic (no organic solvent remnant in extracts)	Advanced and not cost effective	(Zinnai et al., 2016)

This thesis explores suitability of novel *Schizochytrium.sp* to realm the sustainable and cost-effective productions for biodiesel and high-valued compounds. Using the research, it delves into the evaluating the biodiesel suitability in *Schizocytrium.sp* strain of biomass, extraction and proposes innovative strategies to optimize productivity. Over the last decade,

thraustochytrids have been a popular research area owning to the wide spectrum of biomolecules that they can produce. The application of these biomolecules in several fields such as pharmaceuticals, feed, and biofuels has evidenced the promising future of these marine heterokonts. Yet, to fully exploit the advantages of thraustochytrids, further research is encouraged to focus on the optimization of microalgae biomass and solvent system used for extractions. The ongoing need for affordable and sustainable fuel sources has spurred interest in alternative energy source research. The creation of biofuel from microbial biomass and agricultural waste is seen as a sustainable and renewable form of green energy that has the potential to offset the high energy demand. Reservoirs of FAME include plant-based oils, animal fats, and microbial lipids. FAME can then be used to create biofuels. Plant-based biofuel production is unreliable for producing energy since it depends on a large arable area and adequate irrigation for raw ingredients. Despite the low cost of animal fats, the biodiesel made from them has high viscosity, high pour points, and flash points.

The special fatty acids that are produced by different species/strains of thraustochytrids depend on their growth conditions and feedstocks provided. Hence, the quality of biodiesel is significantly influenced by the fatty acid methyl esters, production process and down streaming processing, hence there are some international standards that are set to balance the monitoring procedure (Faried et al., 2017; Vignesh et al., 2020).In general high saturated fatty acids (SFAs) and monounsaturated fatty acid (MUFAs) are desirable for biodiesel production as their oxidative stability, ignition properties i.e. thermal stability. It is interesting to know that thraustochytrid species contained fatty acid profiles where they had high levels of palmitic acid (C16:0) and low degree of unsaturation which is a vital quality for biodiesels (Lee Chang et al., 2015). Heterotrophic marine protists thraustochytrids have received increasingly global attention as renewable, sustainable and alternative source of biodiesel because of their high ability of saturated fatty acids (SFA) accumulation. However, the influence of various carbon sources (glycerol, glucose, maltose, sucrose), extrinsic factors (nutrients and solvent extractions) on thraustochytrids culture and optimal conditions for high SFAs production are poorly described. In the present study, two thraustochytrid strains, *Schizochytrium.sp* and *M164 Schizochytrium.sp* has been investigated for the suitability of biodiesel production.

1.9.0 Hypothesis

1. It is hypothesized that use of different medium and process parameters can enhance higher SFA yields in an oleaginous microbe.

2. The use solvents (polar and non-polar), and their mixtures may assist in improving lipid yields for biodiesel purposes.

1.8 Aims

1. Media optimization for M164 strain of *Schizochytrium.sp,* to enhance SFA (saturated fatty acid levels).

2. Developing an effective downstream method for extracting lipids for its usage in biodiesel and high value compounds.

Chapter 2

2 Materials and Methods

2.1.0 Chemicals

All chemicals used in this research were of analytical grade. Medium components that include glucose, yeast extract, peptone, were ordered from Sigma-Aldrich, USA. Sea salt (Instant Ocean, France) and MiliQ water were used for preparation of media for biomass production. Following to solvent extraction; chloroform (from Merck), and methanol (from Sigma-Aldrich, USA) were used in the lipid extractions. Carbon sources (glucose, glycerol, maltose, sucrose) were ordered from Sigma-Aldrich, USA. Acetone, HPLC grade solvent (Hexane), acetyl chloride was used for fatty acid methyl esters standard mixture.

Table 1 Carefully conducted experiments considering the hazards and response to be taken for all the necessary precautions. The training and safety inductions included workshops on managing the risk and was provided by my co-supervisor Dr Mariam, Ms Kushari Burns and Ms Shweta Sahni

Hazard	Pictogram	Response
Chloroform		IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower. IF INHALED: Remove person to fresh air and keep comfortable for breathing. Immediately call a POISON CENTER or doctor/ physician. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or doctor/ physician. Absorb spillage to prevent material damage
Methanol		
Toluene		
Acetyl chloride		
Hexane		

2.2.0 Biomass cultivation of Thraustochytrids

A *Schizochytrium.sp* (S2 wildtype) *Schizochytrium.sp* strain (M164, a mutant obtained through ARTP-mutagenesis was provided for making a comparison) was sourced from in house collection of the Bioprocessing Laboratory, Medical Biotechnology, College of Medicine and Public Health, Flinders University. This S2 strain was isolated by Dr. Adarsh Gupta (Post-Doctoral researcher working with Associate Professor Munish Puri). Thraustochytrid strain (S2) used in this study was maintained on GYP medium containing (g/L) glucose 10, Yeast Extract 1, Peptone 1, agar 10 and 50% of artificial seawater and miliQ water at 30°C Seed medium (50 ml) contains glucose 10, Yeast Extract 1, Peptone 1 was inoculated from agar plates and grown for 2 days in a shake flask at 30°C, at 180rpm (Gupta et al., 2013). After 2 days, inoculum (10%) was used to inoculate the production media which contains glucose 10, Yeast Extract 1, Peptone 1 and incubated under the same conditions for 5 days.

2.3.0 Culture maintenance

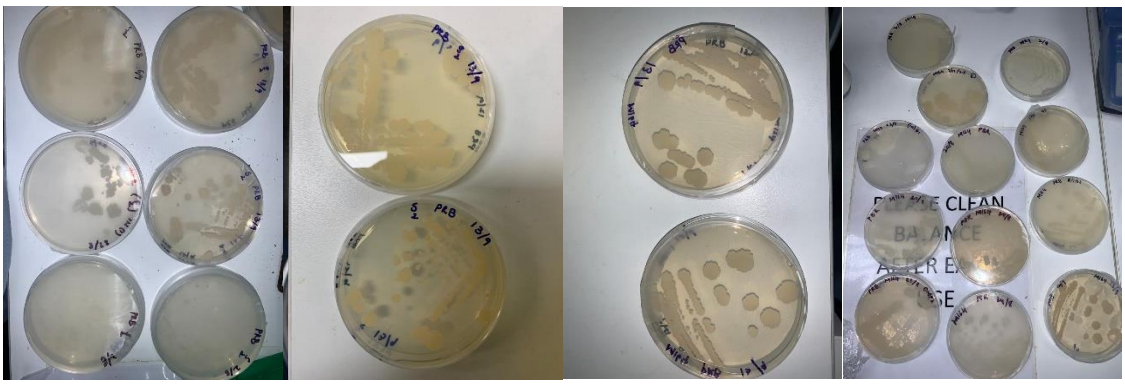


Figure 6 Schizochytrium sp. 164 (putative mutant) and Schizochytrium sp. (wildtype) culture maintained in Agar plates for treatment with various carbon sources.

The microbial culture was maintained on agar plates with following composition: yeast 2 gL^{-1}, agar 15gL^{-1}, peptone 2 gL^{-1}, and glucose 5 gL^{-1}as well as ASW/MilliQ (50/50). Used sterile petri plates and carefully platted in biosafety zone under controlled conditions and kept the plates upside down for better air circulation. This was labelled with dates and strain platted. To avoid contamination the plate was used within 2 weeks and for cells to stay alive it was sometimes stored in -80˚C. Agar culture media is typically used in petri dishes or larger, smaller vessels for microalgal culture, such as 50 mL to 100 mL tissue culture flasks. Prior to moving

the microalgae culture to a bigger growing scale, this stage can also be utilised to preserve and maintain it. To achieve successful growth, it is generally recommended to inoculate and transfer around 10–20% of the stationary phase microalgal biomass into a fresh batch culture medium (i.e., preculture stage). More details and an emphasis on the growth processes, including autotrophic (photoheterotrophic and photoautotrophic), heterotrophic, and mixotrophic growth modes, are given in the next section.

Before conducting the experiment, the strains were checked for any contaminations under the microscope at 40X objective for the inoculum and then carefully transferred to fermentation medium as per the compositional medium. There was dilution of the fermentaion media for obtaining readings, after dilutions and blank readings was obtained. The dilution factor is 10^{-9}. The spectroscope was adjusted to 660 nm and using cuvettes of 4.5 ml, it was carefully obtained for the readings OD.

2.4.0 Morphological observation

Figure 7 -Morphological observation of culture plates of Schizochytrium.sp M164 *under naked eye visualization, it appears to be* brighter yellow compared to Schizochytrium.sp (wildtype) in appearance.

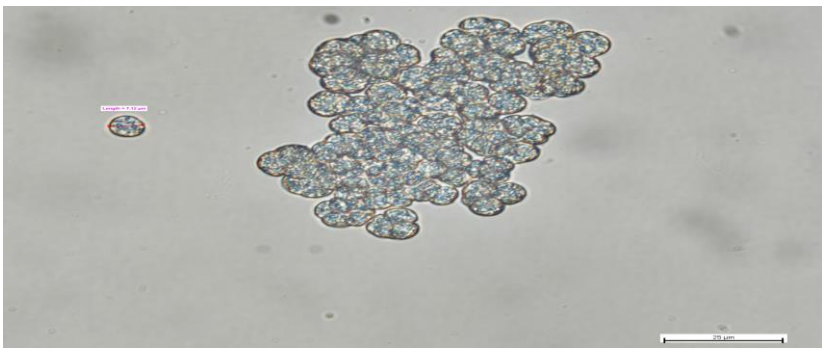


Figure 8- *Schizochytrium sp. (S2)* under 40X objective containing active cells from inoculum with 48 hours of incubation period. The scale used for cell size determination is 25µm to maintain uniformity for having a base comparison of cell size differences from wildtype/parent type to mutant strain.

2.5.0 Biomass analysis

Schizochytrium sp. fermentation was carried in a 20 ml fermentation medium contained in a 100 ml Erlenmeyer flask for 7 days. The samples (2 ml) were withdrawn from the fermentation medium at regular intervals of time (24 h) for 5 days. The sample was centrifuged (3500 rpm for 10 min) and the supernatant was removed for biomass analysis. The remaining pellet was freeze-dried and weighed to find the final weight of the biomass. Freeze-dried biomass was stored at -20°C for further use.

Inoculation: To the prepare the fermentation media, glucose (8% from 40 % glucose stock) was added in the flask. To 30 ml of ASW and 30 ml Mili Q set to heat at 50°C dissolve 40 g of glucose. This was made up to a total volume of 100 ml with 50/50 ASW/Mili Q mixture. Later this mixture was sterilized by the glucose solution by filtering into a sterile 50 ml falcon tube in the biosafety cabinet. After covering the solution, this was sealed with parafilm and stored at 4°C.After which this we homogenized the inoculum (used an automated pipette set to 1 ml and taken out and dispensed the sample several times until homogenised throughout). Without allowing the mixture to settle we added 2 ml of inoculum culture to each fermentation flask.

Sample collection was performed for biomass, lipid, and growth curve analysis as follows: Biomass-In a pre-weighed 15/50 ml falcon tube depending on the experimental set up, added

10/50 ml of homogenized sample. Preserved aliquot as needed were used. Centrifuged the collected sample at 3500 pm for 5 min, decanted the liquid. Washed the biomass with 10 ml of Mili Q water and centrifuged at 3500 pm for 5 mins. Later this was decanted from the water and disposed by adding sodium hypochlorite, the collected biomass added a piece of aluminum foil and piece holes for vapor exchange. We freeze samples at -80°C for 1 h and freeze dried for 48 h. Weighed the sample and determined the biomass (gL^{-1})(Huang et al., 2014).

2.6.0 Lipid Extraction

Freeze-dried cells (10 mg) were taken in a 1.5 ml centrifuge tube to which 600 µl of chloroform: methanol (2:1) is added. The tube was vortexed for 2 min before centrifugation at 10,000 rpm for 10 min. The extraction was repeated three times. The supernatant was collected in glass vials (after filtration with 0.22 µm filter). Collected solvent supernatant was dried in Dry Block Heater (Ratek instruments) at 50°C and the lipid weight was measured gravimetrically based on a protocol (Gupta et al., 2016).

2.7.0 Preparation of fatty acid methyl esters

For analysis, lipids were changed into FAMEs in accordance with (Gupta et al., 2013). To create the internal standard solution, 50 mg of methyl non-adecanoate were dissolved in 10 ml of toluene. To make an antioxidant solution, 100 mg of butylated hydroxytoluene (BHT) were dissolved in 100 millilitres of toluene. Acetyl chloride (1 ml) was added dropwise to methanol (10 ml) and mixed on ice for one hour to create acidic methanol. The extracted lipid (5 mg) was diluted in 500 µl of toluene, then acidic methanol (500 µl), an antioxidant solution (200 µl), and an internal standard (10 µl) were added. This was incubated for 24 hours at 50 °C in a sealed glass vial. Following cooling, 1 ml of a 5% sodium chloride solution was added, and the liquid was then moved to a glass vial. FAMEs were extracted into 1 ml of hexane, and the resulting hexane layer was then moved to a fresh glass vial. After washing with 1 millilitre of 2% potassium bicarbonate solution, the hexane layer was moved to a fresh glass vial. After adding sodium sulphate to eliminate surplus moisture, 450 µl was placed in a GC vial for analysis using Shimadzu's Gas-Chromatography with Flame Ionisation Detector (GC-FID).

Figure 9- Experimental set up in biosafety cabinet at sterilised conditions after 15 minutes of UV Exposure in time frame and 70% ethanol wipe to maintain culture growth without cross contaminants and other environmental contaminants.

2.9.1 Overview of the methodology adapted to achieve higher SFA (Aim1 and 2 of the study)

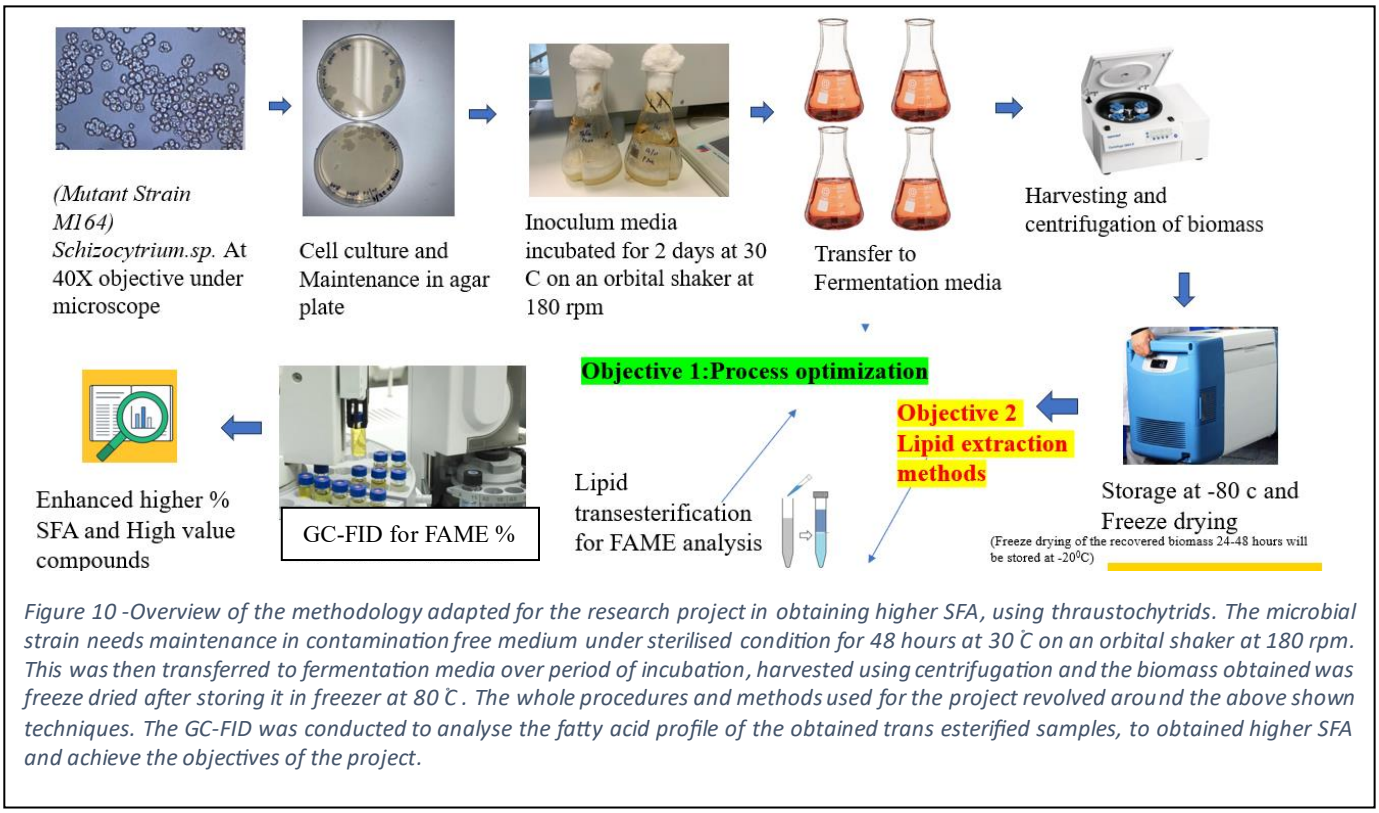


Figure 10 -Overview of the methodology adapted for the research project in obtaining higher SFA, using thraustochytrids. The microbial strain needs maintenance in contamination free medium under sterilised condition for 48 hours at 30 ̊C on an orbital shaker at 180 rpm. This was then transferred to fermentation media over period of incubation, harvested using centrifugation and the biomass obtained was freeze dried after storing it in freezer at 80 ̊C . The whole procedures and methods used for the project revolved around the above shown techniques. The GC-FID was conducted to analyse the fatty acid profile of the obtained trans esterified samples, to obtained higher SFA and achieve the objectives of the project.

Chapter 3

3.0.0 Results and Discussion

3.1.0 Growth profile

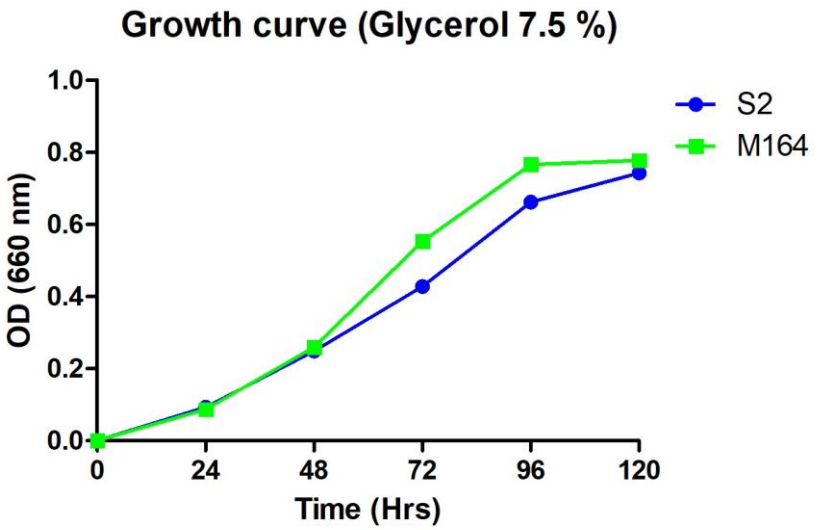


Figure 11-Represents the Growth curve for Schizochytrium.sp and Schizochytrium.sp (164) using glycerol 7.5% of fermentation media at different time intervals. The optical density was measured at 660 nm using UV spectroscope and understand the growth kinetics of individual strain (The graph was generated using Graphpad prism tool.)

Growth curve was plotted based on the results obtained from the experiment performed on *Schizochytrium.sp* (S2) and *Schizochytrium.sp (164)* in a glycerol (7.5%, v/v) containing fermentation medium (GYP) as presented in the figure 11. *Schizochytrium* M164 can

proliferate at a higher growth rate in 7.5 % glycerol media, at controlled conditions (this includes 30 ^{0}C, at 180 rpm in seed medium). when compared with wildtype (Fig 10). From day 1 to day 5, exponential increase in growth of M164, was observed when compared to S2.

3.2. 0 Various parameters that influence the cultivation of heterotrophic marine microalgae

For a microalga to grow efficiently, it is essential to provide process conditions based on which biological activity is promoted. Microalgae growth can be inhibited or disturbed by temperatures that are either below or above the optimal range, which eventually impacts the total amount of biomass produced. While low temperatures can affect the kinetics of enzymes, high temperatures have the potential to inactivate proteins. Most microalgae have been found to thrive best at temperatures between 20 and 30 degrees Celsius, with the exact range varying depending on the region in which they are found (Brindhadevi et al., 2021). Nonetheless, Khoo et al. (2020) report that thermophilic algae can flourish at 80°C.

3.2.2 pH

Just like other variables, pH is essential to growth. The majority of microalgal species grew rapidly between pH 6 and 8.76 (Andrade et al., 2021). With a pH range of 4–10, *Chlorella vulgaris* has been shown to grow best in a pH of 9–10. According to Khoo et al. (2020), fungal contamination can be avoided at acidic conditions of pH 3–4. pH values between 4 and 8 can be used to cultivate different strains of thraustochytrids. According to Pawar et al. (2021), the species *Aurantiochytrium limanicum* had the maximum biomass (13.69 g/L) and lipid content (52.34%) when cultivated at pH values between 6 and 7. Consequently, this indicates that pH is essential for the growth of microalgal cells.

3.2.3 Mixing

The mixing of medium components is crucial for the cultivation of microalgae. In a microbial culture, it is essential to evenly combine all the nutrients, temperature, and pH since this prevents sedimentation and clumping and gives light where it is needed (Khoo et al., 2020).

Furthermore, the growth of microalgal biomass may be inhibited by elevated quantities of dissolved oxygen (Kazbar and colleagues, 2019).

3.2.4 Nutrients

Microalgae needs different nutrients at specific ratios for their growth. For example, sodium bicarbonate and CO_2 are needed for autotrophs as their only carbon source. Starch, glycerol, glucose, sucrose, and nitrogen sources are required for the heterotrophs whereas combined carbon sources are used in case of mixotrophs. Hence, it is very important to provide all the nutrients at optimum pH and temperature (Khoo et al., 2020).

3.2.7 Effect of various carbon sources

Schizochytrium.sp M164 biomass was obtained when grown different glycerol concentrations (3%, 5%, 7.5%, 10% and 12.)It has been commonly documented that adding a carbon source to the medium increases the growth and lipid content of microalgae (Qiao & Wang, 2009). The use of various carbon sources gave the importance of cost or low carbon source required to meet the larger volumes for preparation of biodiesel –*Schizochytrium.sp*, a heterotrophic microbe, requires organic carbon substrate (such as glucose, glycerol, fructose, sucrose, maltose, and molasses)for its growth. Since it makes use of a free natural resource, in the case of most production systems—it is seen as a viable strategy for the large-scale production of algal biomass (Khoo et al., 2023).

The biomass yield increases as more glucose is supplied in the fermentation medium (Fig12). Most of the carbon sources that are preferred by living cells on Earth are glucose. Furthermore, it has been observed that in numerous heterotrophic cultures of microalgae, glucose serves as a source of carbon and energy (Cheng et al., 2009). The biomass obtained from glycerol 7.5% concentration is 27.3 g/l, which is the highest when compared to all other concentartions.M164 strain is utilizing the nutrients well in glycerol as carbon source. When cultivating particular strains like *Schizochytrium.sp* (S2 parent strain) and *Schizochytrium*.sp, (164) glycerol is crucial. Also, M164 strain is having ability to readily metabolize glycerol due to its simple conversion to acetyl-CoA, a crucial precursor for lipid biosynthesis. This is crucial for *M164 Schizochytrium*.sp in particular because of its high lipid content, which is useful for a variety of processes like the extraction of omega-3 fatty acids and the manufacture of biodiesel

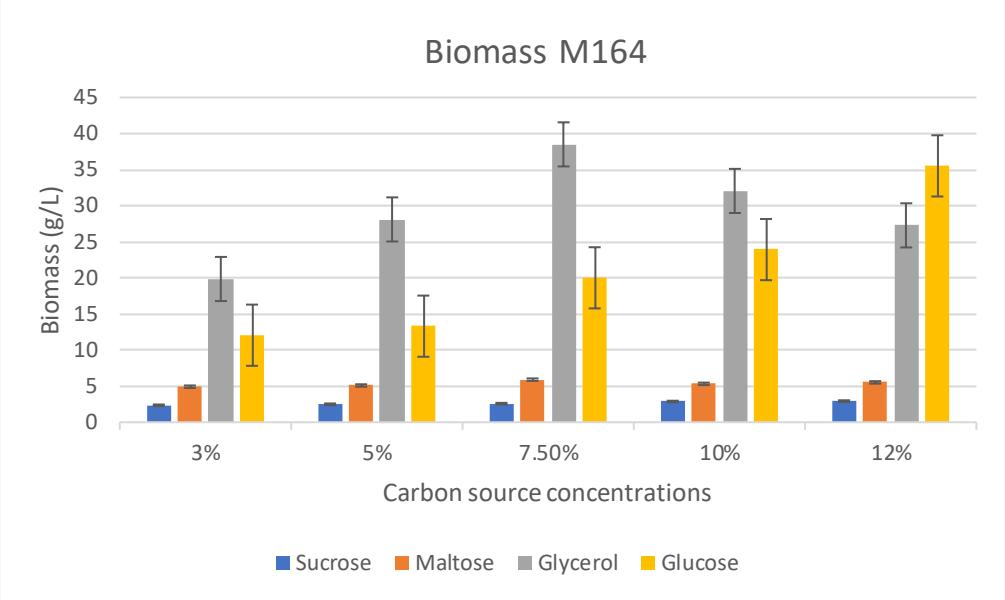


Figure 12- Comparison of different concentrations of carbon sources such as glycerol, glucose, maltose and sucrose investigated for improving biomass growth. This experiment was conducted at conditions which include 180 rpm, 30℃ for 5 days of incubation period.

3.2.8 Importance of process optimization

The process optimization for lipid synthesis is presented in figure 10. The selection and cultivation of the strains is the first stage, followed by harvesting and lipid extraction. Lipids are extracted utilising a variety of techniques, as detailed from the harvested dried biomass. SFAs make up higher % of the lipids in the cell bodies of microalgal cells, with the remaining are mono unsaturated and PUFAs containing varying proportions of free fatty acids as well (Table 2). According to Milledge et al. (2014), various microalgal species have varying lipid contents that fluctuated from 2.40 and 62.0% dry weight basis. *Scenedesmus* sp. with *Chlorella vulgaris* containing sodium haemapodous produced the lipid content of 14.0–18.5 percent, 20%, and 11.0-43.0% of dry (Deshmukh et al., 2019) biomass. Dry cell mass and DHA synthesis in *Schizochytrium.sp* Mangrovei G13 was 14 g/L and ASW when sodium sulphate (20 g/L) was substituted. At 107 hours, 2.17 g/L of biomass was observed (Shene et al., 2010). The effects of polar (acetone/methanol) and non-polar (chloroform/hexane) solvents on the lipid yield, composition of fatty acids methyl esters (FAMEs), and biodiesel characteristics of microalgae are reported in many works. Hexane and chloroform extracted a larger yield of lipids (100.01 and 94.33 mg/g) than acetone and methanol (40.12 mg/g), 86.91 mg/g as well. The composition of FAME in microalgal lipids was similarly influenced by the polarity of the

solvents. Entire saturation by using chloroform, the extracted lipids' saturated and unsaturated fatty acids were found to be 61.53% and 38.47%, respectively (Zarrinmehr et al., 2022).

3.2.10 Effect of different solvents on lipid yields

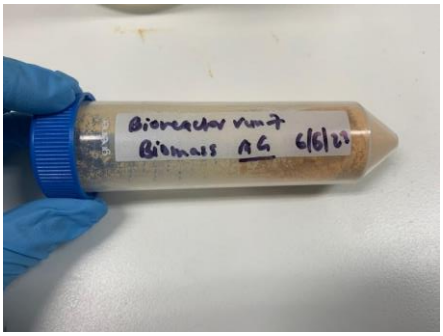


Figure 13-Freeze dried sample of Schizochytrium.sp S4 used for lipid extractions

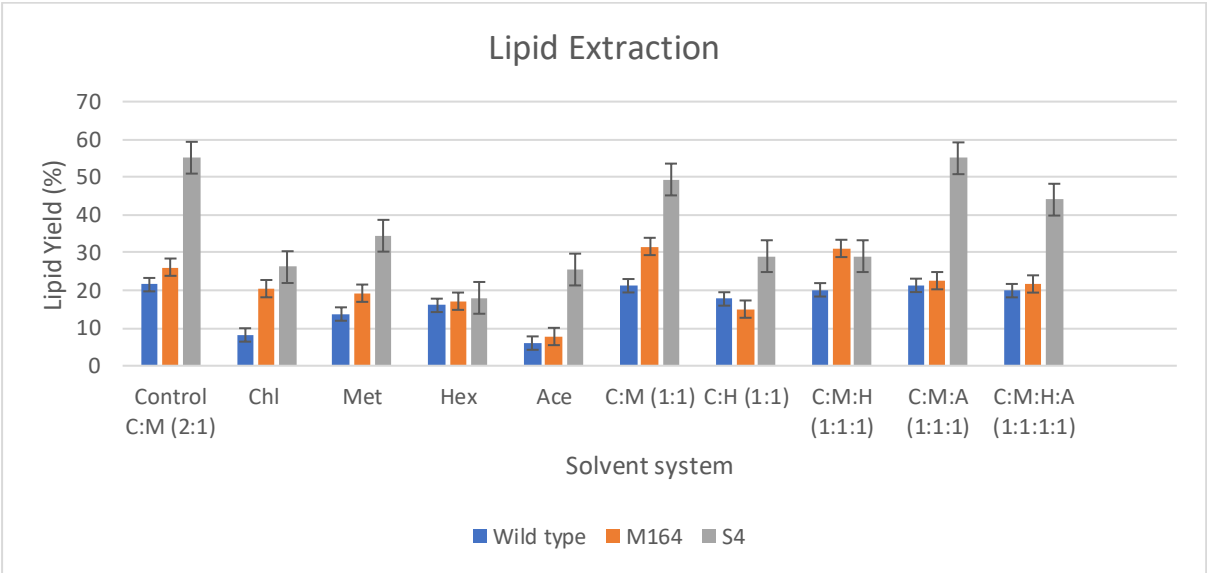


Figure 14- Lipid extraction using Schizochytrium.sp (Wildtype), Schizochytrium.sp, (S4) and Mutant Schizochytrium.sp,(M164) biomass with different solvent systems.

Literatures studies suggest that a cost-effective cell disruption technique should be optimised to extract high-quality lipids from microbial cells. Because the extraction solvent has better access to fatty acids as a result of cell rupture, microalgae release more intracellular lipids (McMillan et al., 2013). Lipid extraction yields from various microalgae are affected by cell disruption techniques as ultrasonication, microwave, bead milling, drying, and supercritical

fluid extraction. In order to effectively extract lipids from a thraustochytrids strain, this study tested different organic solvents and mixtures. Although microalgae have been highlighted as a promising feedstock for biodiesel production, biodiesel from microalgal lipids needs to meet the required quality authorized by international standards such as European standards (EN 14214) and American Society for Testing and Materials (ASTM D6751) (de Jesus et al., 2020; Vignesh et al., 2020).

The fatty acids of microalgae are divided into saturated (without double bonds), monounsaturated (one double bond) and polyunsaturated (more than one double bonds) fatty acids with a 12–22 linear carbon length (Wu and Miao, 2014). The properties of biodiesel are highly contingent on the degree of saturation and a carbon length of fatty acid (Deshmukh et al., 2019). Saturated fatty acids provide storage stability to the biodiesel and protect it from autooxidation during a long-term storage, while unsaturated fatty acids with lower viscosity are beneficial in terms of cold flow characteristics (Wu and Miao, 2014).

3.3.0 Use of solvent extractions

The methodology followed is presented in the (Fig-19). Here we have used individual solvents, binary solvents, tertiary solvents, and quaternary solvents to understand the permeability of solvents in terms of the cell membrane capacity to disrupt the cell wall of the same species. Based on the conducted experiment, results have shown that optimized binary solvent that is chloroform and methanol in combination 1:1 ratio as resulted in high lipid yield.

In situ transesterification can sometimes benefit from the use of co-solvents, which improve the solubility of the alcohol employed in the process and speed up the reaction rate (Go et al., 2016). Water is known to hinder the extraction and transesterification of lipids, making it particularly crucial to avoid mixing alcohol and water to create a homogeneous mixture. Consequently, adding a co-solvent during the process allows for the extraction of more lipids and increases the mass transfer of the reactant (Zhang et al., 2015). Co-solvent, however, is not involved in the reaction that occurs between the two. As a result, the co-solvent concentration would not change before or after the reaction. For lipid extraction and in situ transesterification of microalgae biomass, chloroform is known to be a highly effective co-solvent (Im et al., 2014).

sHowever, chloroform's superior co-solvent property is conditional, meaning it depends on the specifics of the procedures followed. According to research done by Najaf Abadi et al. , the best FAME yield was achieved when hexane was used as the co-solvent, with an ideal ratio of hexane to biomass of 6 to 1. This study examined the efficacy of various co-solvents for in situ transesterification of Chlorella vulgaris using supercritical methanol. In the same study, however, the use of chloroform really produced a lower FAME production than the control. Thraustochytrids are heterotrophic fungi like organisms ubiquitously found in the marine environment. Molecular phylogenetic studies revealed that they are classified under the class labyrinthulomycetes, phylum Heterokont within the kingdom Chromistan (Chang et al., 2012). Ultra-structurally, they show a close relationship with heterokont algae because of the presence of unequal flagella. These species are regarded as a promising producer of omega 3 fatty acids including DHA and EPA (Sun et al., 2017). To enhance the biomass and oil accumulation in microalgae various substrates are used such as glucose (1.44 g/l), glycerol (raw) (8.32 g/L) ,sucrose xylose (0.48 g/L),standard cellobiose (0.38 g/L), sugar hydrolysates (1.8 g/L) (Gupta et al., 2013), agricultural wastes such as rice straw (Joe et al., 2015), sugarcane bagasse hydrolysates provides (10.45 g/L) dry biomass whereas 45.15% of lipid content was obtained after the 72 h cultivation (Nguyen et al., 2018). Because of the presence of various hydrolytic enzymes, these species are considered as highly adaptive organisms (Xie et al., 2017). This diversified feature sparkled the research and explore the various biotechnological applications to produce a wide range of bioactive compounds such as DHA and EPA (Chang et al., 2011)

3.3.1Growth curve for S2 and M164 using glucose

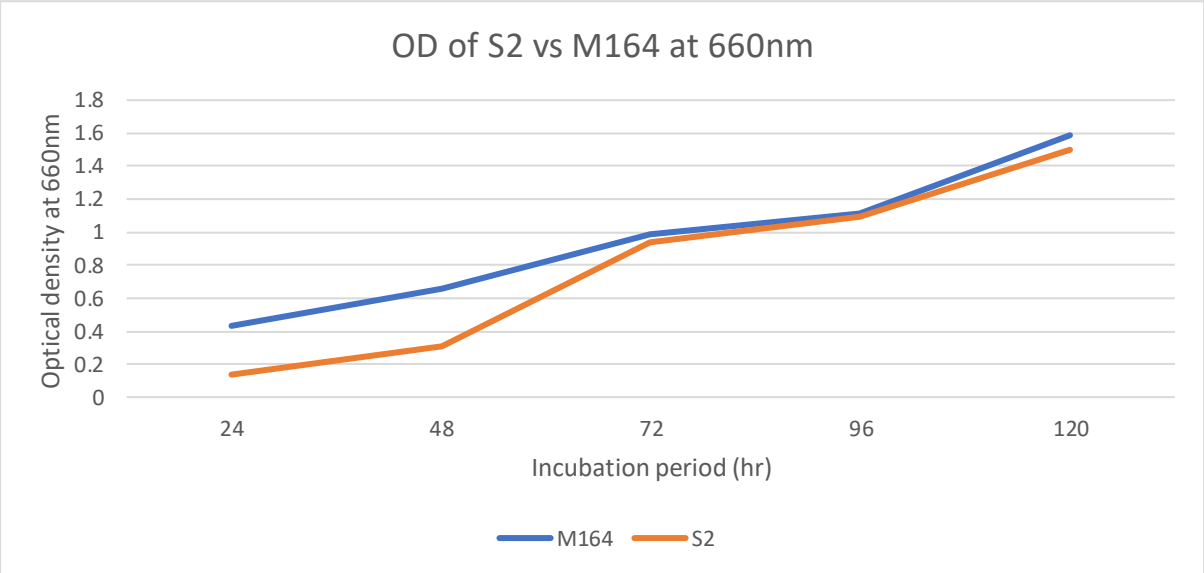


Figure 15 Optical density of S2 and M164 at 660nm under glucose 8% concentration at 30℃, 180 Rpm (GYP media)

To achieve the best microalgae output, it is essential to keep a close watch on the algae's growth. The increase in biomass concentration was observed from the growth curve of *Schizochytrium* which was measured by optical density at 660 nm, biomass and lipid yield. The cell counting (microscopic) approach (Politaeva et al., 2017), dry cell weight (DCW) measurement (Maurya et al., 2016), and optical density (OD) measurement are frequently used in the literature to track the growth of microalgae (Nielsen & Hansen, 2019).

3.3.1 Lipid Yield

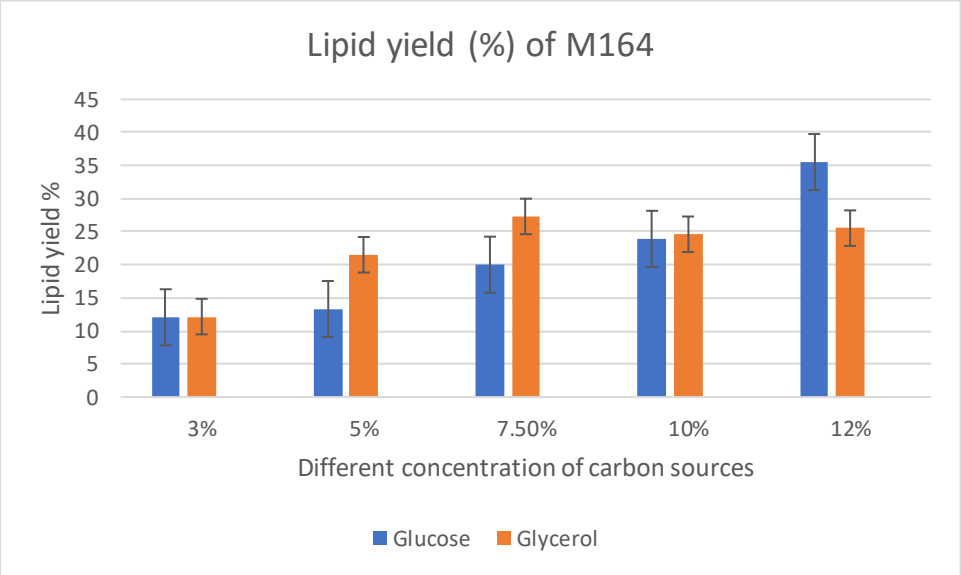


Figure 16 -Lipid yield from Schizochytrium.sp in glucose and glycerol medium

$$Total\ lipid\ content\ (\%) = \frac{Weight\ of\ dried\ lipids\ (mg)}{Weight\ of\ dried\ biomass\ (mg)}\ x\ 100\%$$

Figure 17- Formula used to calculate the total lipid content

To achieve this objective, various carbon sources at different concentrations (3%-12%, w/v) including glucose (a simple sugar), glycerol (a compound found in fats/oils), maltose

(disaccaharide), and sucrose (table sugar) were tested in the fermentation medium. Results showed that when glycerol was used as the carbon source,a maximum biomass yield of 27.3 grams per liter (L) was obtained.The M164 strain exhibited a higher lipid yield compared to glucose-based cultures; specifically a lipid content of 21.91% (Fig 21). In contrast, the wild-type (WT) strain yielded 24 .29 g/L of lipids and a biomass yield of 23.92 g/L in the presence glucose as a carbon source, resulting in a lipid content of 24.26% (Fig-20 a)

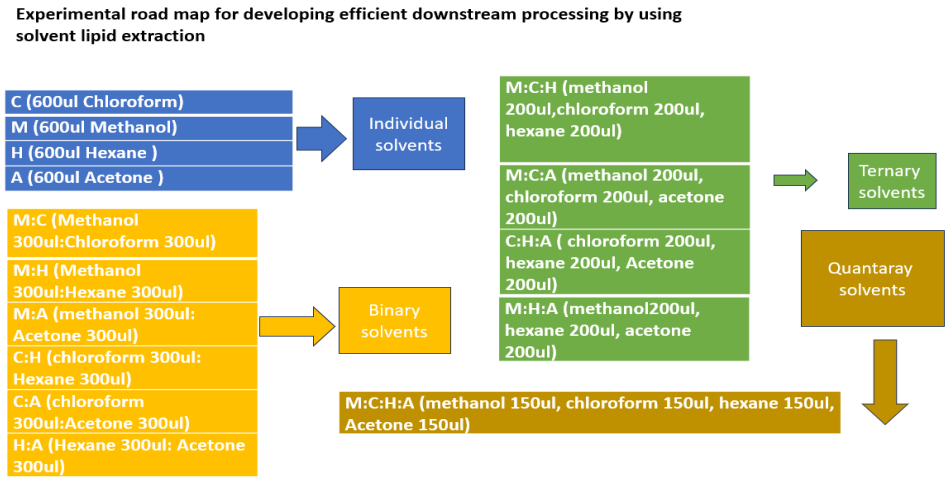


Figure 18- Pictorial representation of experimental flowchart

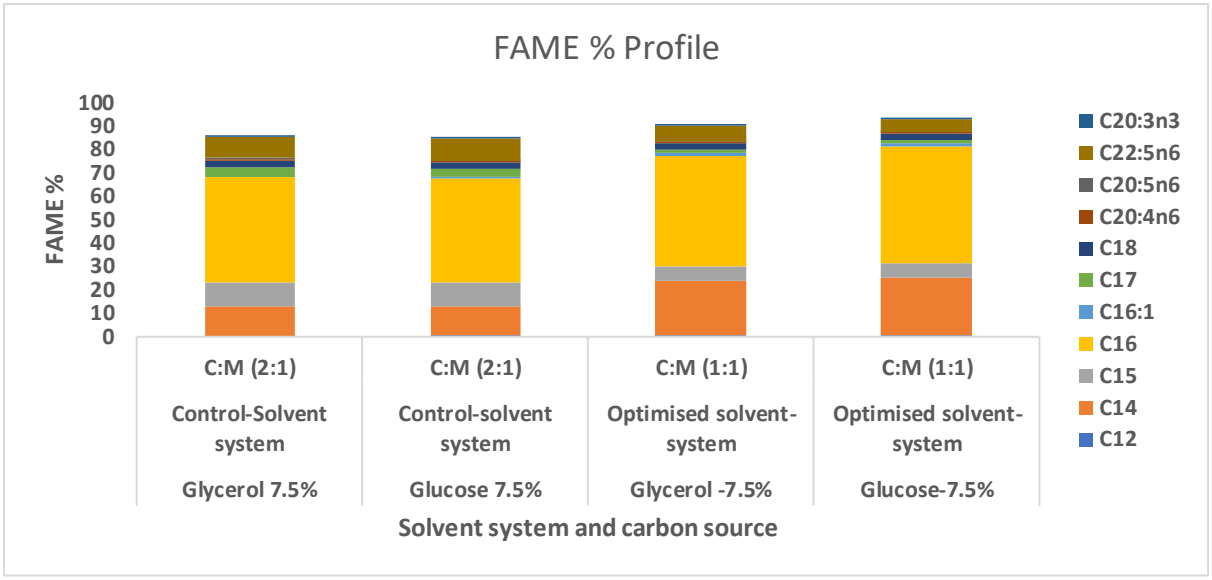
Table 2

3.3.2 Fatty acid methyl ester analysis of lipids (FAME analysis)

FAME %	Glycerol 7.5% Control-Solvent system C:M (2:1)	Glucose 7.5% Control-solvent system C:M (2:1)	Glycerol -7.5% Optimised solvent-system C:M (1:1)	Glucose-7.5% Optimised solvent-system C:M (1:1)
C12	0.05	0.33	0.79	0.87
C14	13.05	12.70	23.41	24.18
C15	10.35	9.88	5.63	6.16
C16	44.80	45.00	47.67	50.31
C16:1	0.37	0.42	1.23	1.27
C17	3.78	3.46	1.29	1.31

C18	2.69	2.60	2.76	2.83
C20:4n6	0.85	0.67	0.71	0.43
C20:5n6	0.42	0.51	0.29	0.27
C22:5n6	9.37	9.36	6.56	5.40
C20:3n3	0.28	0.37	0.30	0.27
CDW gL^{-1}	27.90	27.90	23.92	23.92
Lipid %	22.15	24.27	21.91	27.2
SFA%	74.72	88.47	80.27	85.66
PUFA%	10.50	10.23	17.21	13.07
MUFA%	0.37	0.42	2.53	1.27

Fatty acids such as myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), EPA (C20:5 Eicosapentaenoic acid), DPA (C22:5, Docosapentaenoic acid), and DHA (C22:6, docosahexaenoic acid) as observed in a fatty acid profile of the *Schizochytrium.sp*



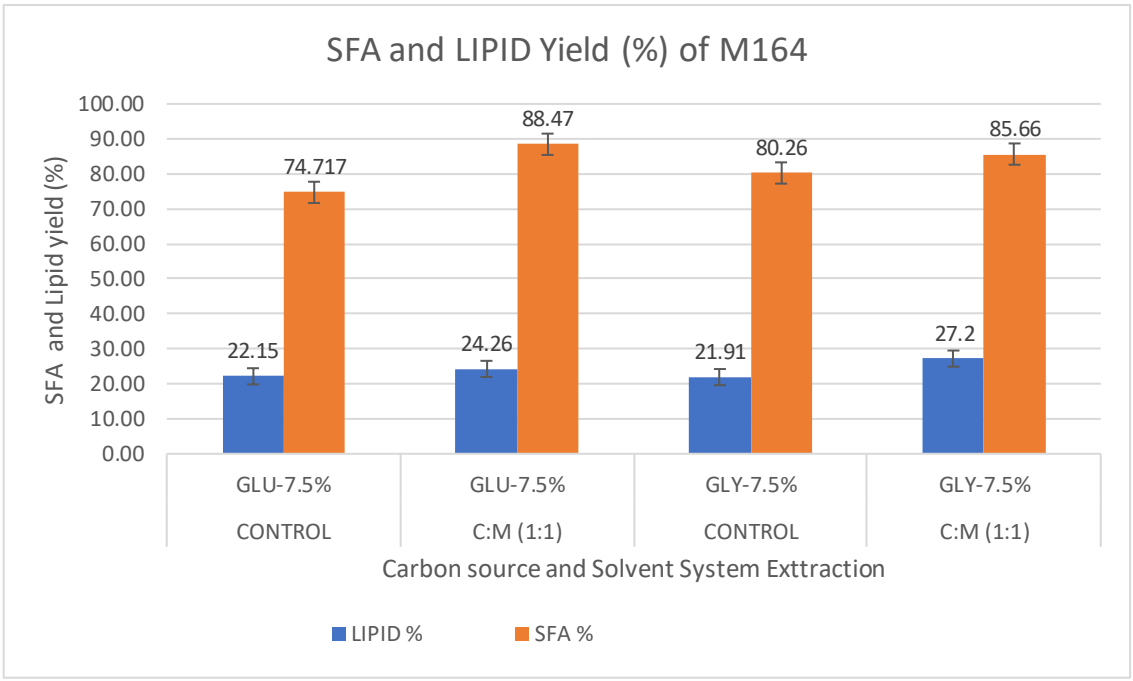


Figure 20 a and b: ***SFA and Lipid yield under different carbon source***

A lipid yield of 22.15 % was obtained when a solvent (2:1) was used for the extraction of biomass grown in a fermentation medium containing glucose (7.5%). Whereas, a solvent ratio (1:1) resulted in a 24.26 % of lipid yields (Fig 21 a and b). The solvent ratio influenced the SFA content.

The quality of the produced biofuel is governed by the quality and content of lipids in the microalgae (Ali et al., 2021) For example, *Tetraselmis maculata* has a total lipid content of less than 4.5%, while for *Schizochytrium* sp. this is greater than 80% (Fig 20; Huerlimann et al., 2010, Siddiki et al., 2022). Additionally, the amount of lipid present in a given microalgae strain varies

depending on its growth phase, with the lowest yields prevalent for logarithmic growing strains in the late logarithmic phase and static or growing strains in the stationary phase (Hu et al., 2008). Lipid quality and production using microalgae are dependent on environmental factors, such as growing time, nutrient availability, and exposure to lighting (Siddiki et al., 2022).The production of SFA from microalgae lipids can be performed using the transesterification process. Transesterification is the chemical reaction between fat (lipids) with alcohol to produce alkyl esters or biodiesel (Mofijur et al., 2013). The fuel quality is influenced by the structural features of the individual fatty acids. The fuel quality is influenced by the structural features of the individual fatty acids. The quality of SAF to be produced can be controlled by selecting an appropriate microalgae strain with different types of fatty acids, the mixture of the different types of fatty acids through the selection of production organisms, increasing the concentration of desired fatty acids in the lipid, or by the genetic modification (Bwapwa et al., 2017).(Gupta et al., 2016)

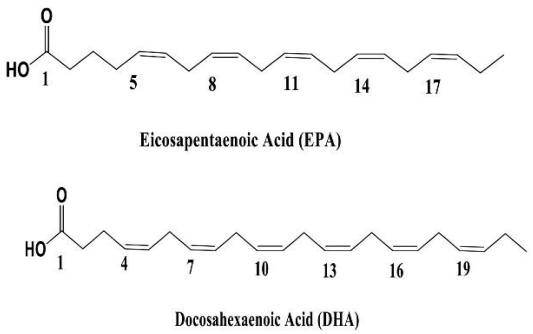


Figure 21 ***Structure of EPA and DHA (Li et al., 2019)***

Polyunsaturated fatty acids (PUFAs) are important nutrients for humans and animals. Microorganisms, such as yeast, filamentous fungi, and microalgae, have successfully been modified to produce PUFAs. Apart from strain improvement and fermentation optimization, efficient and cost-effective downstream processing will determine whether production can advance from the laboratory to the factory. Thraustochytrids, the heterotrophic, marine, straminipilan protists, are now established candidates for commercial production of the omega-3 polyunsaturated fatty acid (ω-3 PUFA), docosahexaenoic acid (DHA), that is important in human health and aquaculture (Fig 21). Extensive screening of cultures from a variety of habitats has yielded strains that produce at least 50% of their biomass as lipids, and DHA comprising at least 25% of the total fatty acids, with a yield of at least 5 g L^{-1}. Most of the lipids occur as triacylglycerols and a lesser amount as phospholipids. Numerous studies have been carried out on salinity, pH, temperature, and media optimization for DHA production.

Commercial production is based on a fed batch method, using high C/N ratio that Favors lipid accumulation. *Schizochytrium* DHA is now commercially available as nutritional supplements for adults and as feeds to enhance DHA levels in larvae of aquaculture animals. Thraustochytrids are emerging as a potential source of other PUFAs such as arachidonic acid and oils with a suite of PUFA profiles that can have specific uses. They are potential sources of astaxanthin and carotenoid pigments, as well as other lipids.

Production of biodiesel from in expensive carbon sources will reduce oil production costs. Glycerol is suggested as the most economical carbon source in microbiological fermentation and is used extensively for growing thraustochytrids. After 5 days, the biomass obtained were (mention biomass of d.5 of S2) g L^{-1} and M164 isolate (mention biomass of D-5 M164) g L^{-1}

3.3.3 Effect of Glycerol on SFA production

The biotechnological potential of thraustochytrids as producers of SFAs that are suitable for biodiesel production. Glycerol has been used based on the available literature finding that it gives higher SFA (saturated fatty acids), which holds significant role in *Schizochytrium*. sp., that are recognised for their capability to accumulate high levels of lipids, that can be converted into biodiesel through transesterification processes (Zhu et al., 2008). The effect of SFA on carbon source such as glycerol for investigating suitability of biodiesel for modulation of lipid biosynthesis which indeed helps to navigate through bio-chemical processes and capacity to produce high value polyunsaturated fatty acids, specially docosahexaenoic acid (DHA), omega 3 fatty acids, making it a promising source for lipid engineered studies.

The fatty acid profile for 7.5% of glycerol (75g/L), gave 74.71% and lipid yield percentage of 22.15% (Table or Fig?) . This particular lipid yield was produced by control solvent system, which include mixture of chloroform and methanol in the ratio (2:1). Its is anticipated that SFA would facilitate biodiesel production from thrastochytrids. Such third generation of biofules can offer various advantages such as reduced greenhouse gas emissions, rapid growth, and productivity, minimal competition to agricultural and food resources.

To verify the ' lipid-extraction properties, the lipid extraction using organic solvents was investigated. According to the adage "like dissolves like," the polarity of the lipid is determined by the presence of fatty acids. As a result, a suitable solvent should be found for the extraction of the entire lipid; however, a universal solvent cannot be used for any microbe with varying fatty acid compositions. Solvent polarity largely determines the

variation in total lipid extraction yields Biodiesel needs to meet international standards such as American society for testing and materials (ASTM).

The fatty acids of microalgae are divided into saturated (without double bonds), monounsaturated (one double bond) and polyunsaturated (more than one double bonds) fatty acids with a 12–22 linear carbon length (Wu and Miao, 2014). The properties of biodiesel are highly contingent on the degree of saturation and a carbon length of fatty acid (Deshmukh et al., 2019). Saturated fatty acids provide storage stability to the biodiesel and protect it from autooxidation during a long-term storage, while unsaturated fatty acids with lower viscosity are beneficial in terms of cold flow characteristics (Wu and Miao, 2014).

Consequently, adding a co-solvent during the process allows for the extraction of more lipids and increases the mass transfer of the reactant (Zhang et al., 2015). Co-solvent, however, is not involved in the reaction that occurs between the two. As a result, the co-solvent concentration would not change before or after the reaction. For lipid extraction and in situ transesterification of microalgae biomass, chloroform is known to be a highly effective co-solvent (Im et al., 2014).

According to research done by Najafabadi et al. , the best FAME yield was achieved when hexane was used as the co-solvent, with an ideal ratio of hexane to biomass of 6 to 1. This study examined the efficacy of various co-solvents for in situ transesterification of Chlorella vulgaris using supercritical methanol. In the same study, however, the use of chloroform really produced a lower FAME production than the control. Thraustochytrids are heterotrophic fungi like organisms ubiquitously found in the marine environment. Molecular phylogenetic studies revealed that they are classified under the class labyrinthulomycetes, phylum Heterokont within the kingdom Chromistan (Chang et al., 2012). Ultra-structurally, they show a close relationship with heterokont algae because of the presence of unequal flagella. These species are regarded as a promising producer of omega 3 fatty acids including DHA and EPA (Sun et al., 2017). To enhance the biomass and oil accumulation in microalgae various substrates are used such as glucose (1.44 g/l), glycerol (raw) (8.32 g/L) ,sucrose xylose (0.48 g/L),standard cellobiose (0.38 g/L), sugar hydrolysates (1.8 g/L) (Gupta et al., 2013), agricultural wastes such as rice straw (Joe et al., 2015), sugarcane bagasse hydrolysates provides (10.45 g/L) dry biomass whereas 45.15% of lipid content was obtained after the 72 h cultivation (Nguyen et al., 2018). Because of the presence of various hydrolytic enzymes, these species are considered as highly adaptive organisms (Xie et al., 2017). This diversified feature sparkled the research

and explore the various biotechnological applications to produce a wide range of bioactive compounds such as DHA and EPA (Chang et al., 2011).

Overall, this study demonstrated the potential of the *Schizocytrium*.sp WT and M164 isolates for production of lipids and other high-value polyunsaturated fatty acids. The findings highlight the importance of optimizing growth conditions, particularly carbon source selection and solvent ratios, in order to enhance biomass production and lipid yields for biodiesel applications

Chapter 4

4.0 Conclusions

Thraustochytrids have gained considerable interest in recent years due to their great potential in producing several high-value compounds. An ideal extraction method would be based on extracting lipids from a microbial biomass grown under optimal culture conditions such as temperature, pH, carbon source, salinity, this study demonstrated the suitability of a novel *Schizochytrium* strain for SFA production. The optimised solvent system resulted in higher SFA 88 % in thraustochytrid. This work shows potential of producing biodiesel fuel from the heterotrophic microalga *Schizochytrium* sp. S2 and *Schizochytrium* sp. 164 when grown in a glycerol-based fermentation medium. Future work may involve the validation of SFA for biodiesel production that meets ASTM standards and control the fuel quality. In addition, devising a novel analytical method for lipid extraction with enhanced selectivity and sensitivity would be preferred.

References

Agency, I. E. (2021). Renewables 2021: Analysis and Forecasts to 2026.

Bahadar, A., & Khan, M. B. (2013). Progress in energy from microalgae: A review. *Renewable and sustainable energy reviews*, *27*, 128-148.

Challagulla, V., Fabbro, L., & Nayar, S. (2015). Biomass, lipid productivity and fatty acid composition of fresh water microalga Rhopalosolen saccatus cultivated under phosphorous limited conditions. *Algal research*, *8*, 69-75.

Dewapriya, P., & Kim, S.-k. (2014). Marine microorganisms: An emerging avenue in modern nutraceuticals and functional foods. *Food Research International*, *56*, 115-125.

Du, Y., Schuur, B., Kersten, S. R., & Brilman, D. W. (2015). Opportunities for switchable solvents for lipid extraction from wet algal biomass: An energy evaluation. *Algal research*, *11*, 271-283.

Faried, M., Samer, M., Abdelsalam, E., Yousef, R., Attia, Y., & Ali, A. (2017). Biodiesel production from microalgae: Processes, technologies and recent advancements. *Renewable and sustainable energy reviews*, *79*, 893-913.

Go, A. W., Sutanto, S., Ong, L. K., Tran-Nguyen, P. L., Ismadji, S., & Ju, Y.-H. (2016). Developments in in-situ (trans) esterification for biodiesel production: A critical review. *Renewable and sustainable energy reviews*, *60*, 284-305.

Günerken, E., D'Hondt, E., Eppink, M., Garcia-Gonzalez, L., Elst, K., & Wijffels, R. H. (2015). Cell disruption for microalgae biorefineries. *Biotechnology advances*, *33*(2), 243-260.

Gupta, A., Singh, D., Barrow, C. J., & Puri, M. (2013). Exploring potential use of Australian thraustochytrids for the bioconversion of glycerol to omega-3 and carotenoids production. *Biochemical engineering journal*, *78*, 11-17.

Gupta, A., Singh, D., Byreddy, A. R., Thyagarajan, T., Sonkar, S. P., Mathur, A. S., Tuli, D. K., Barrow, C. J., & 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.

Halim, R., Rupasinghe, T. W., Tull, D. L., & Webley, P. A. (2013). Mechanical cell disruption for lipid extraction from microalgal biomass. *Bioresource Technology*, *140*, 53-63.

Huang, Y., Hong, A., Zhang, D., & Li, L. (2014). Comparison of cell rupturing by ozonation and ultrasonication for algal lipid extraction from Chlorella vulgaris. *Environmental technology*, *35*(8), 931-937.

Im, H., Lee, H., Park, M. S., Yang, J.-W., & Lee, J. W. (2014). Concurrent extraction and reaction for the production of biodiesel from wet microalgae. *Bioresource Technology*, *152*, 534-537.

Jenkins, W., Jenkins, A., Jenkins, A., & Brydson, C. (2019). *The portfolio diet for cardiovascular disease risk reduction: an evidence based approach to lower cholesterol through plant food consumption*. Academic Press.

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. *International Journal of Renewable Energy Research*, *5*(2), 598-621.

Kadir, W. N. A., Lam, M. K., Uemura, Y., Lim, J. W., & Lee, K. T. (2018). Harvesting and pre-treatment of microalgae cultivated in wastewater for biodiesel production: A review. *Energy Conversion and Management*, *171*, 1416-1429.

Khoo, K. S., Ahmad, I., Chew, K. W., Iwamoto, K., Bhatnagar, A., & Show, P. L. (2023). Enhanced microalgal lipid production for biofuel using different strategies including genetic modification of microalgae: A review. *Progress in Energy and Combustion Science*, *96*, 101071.

Kiss, A. A., & Bildea, C. S. (2012). A review of biodiesel production by integrated reactive separation technologies. *Journal of Chemical Technology & Biotechnology*, *87*(7), 861-879.

Kumar, S. J., Kumar, G. V., Dash, A., Scholz, P., & Banerjee, R. (2017). Sustainable green solvents and techniques for lipid extraction from microalgae: A review. *Algal research*, *21*, 138-147.

Lee Chang, K. J., Dunstan, G. A., Abell, G. C., 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*, 2215-2231.

Lee Chang, K. J., Rye, L., Dunstan, G. A., Grant, T., Koutoulis, A., Nichols, P. D., & Blackburn, S. I. (2015). Life cycle assessment: heterotrophic cultivation of thraustochytrids for biodiesel production. *Journal of Applied Phycology*, *27*, 639-647.

Li, X., Liu, J., Chen, G., Zhang, J., Wang, C., & Liu, B. (2019). Extraction and purification of eicosapentaenoic acid and docosahexaenoic acid from microalgae: A critical review. *Algal research*, *43*, 101619.

Li, X., Xu, H., & Wu, Q. (2007). Large-scale biodiesel production from microalga Chlorella protothecoides through heterotrophic cultivation in bioreactors. *Biotechnology and bioengineering*, *98*(4), 764-771.

Manandhar-Shrestha, K., & Hildebrand, M. (2013). Development of flow cytometric procedures for the efficient isolation of improved lipid accumulation mutants in a Chlorella sp. microalga. *Journal of Applied Phycology*, *25*, 1643-1651.

Maurya, R., Paliwal, C., Chokshi, K., Pancha, I., Ghosh, T., Satpati, G. G., Pal, R., Ghosh, A., & Mishra, S. (2016). Hydrolysate of lipid extracted microalgal biomass residue: An algal growth promoter and enhancer. *Bioresource Technology*, *207*, 197-204.

McMillan, J. R., Watson, I. A., Ali, M., & Jaafar, W. (2013). Evaluation and comparison of algal cell disruption methods: Microwave, waterbath, blender, ultrasonic and laser treatment. *Applied Energy*, *103*, 128-134.

Nautiyal, P., Subramanian, K., & Dastidar, M. (2014). Production and characterization of biodiesel from algae. *Fuel Processing Technology*, *120*, 79-88.

Newell, R. G., Qian, Y., & Raimi, D. (2016). *Global energy outlook 2015*.

Nielsen, S. L., & Hansen, B. W. (2019). Evaluation of the robustness of optical density as a tool for estimation of biomass in microalgal cultivation: The effects of growth conditions and physiological state. *Aquaculture research*, *50*(9), 2698-2706.

Pinzi, S., Leiva, D., López-García, I., Redel-Macías, M. D., & Dorado, M. P. (2014). Latest trends in feedstocks for biodiesel production. *Biofuels, Bioproducts and Biorefining*, *8*(1), 126-143.

Politaeva, N., Kuznetsova, T., Smyatskaya, Y., Trukhina, E., & Ovchinnikov, F. (2017). Impact of various physical exposures on Chlorella Sorokiniana microalgae cultivation. *International Journal of Applied Engineering Research*, *12*(21), 11488-11492.

Sadeghinezhad, E., Kazi, S., Sadeghinejad, F., Badarudin, A., Mehrali, M., Sadri, R., & Safaei, M. R. (2014). A comprehensive literature review of bio-fuel performance in internal combustion engine and relevant costs involvement. *Renewable and sustainable energy reviews*, *30*, 29-44.

Sandani, W. P., Nishshanka, G. K. S. H., Premaratne, R. G. M. M., Wijayasekera, S. C. N., Ariyadasa, T. U., & Premachandra, J. K. (2020). Comparative assessment of pretreatment

strategies for production of microalgae-based biodiesel from locally isolated Chlorella homosphaera. *Journal of bioscience and bioengineering*, *130*(3), 295-305.

Schüler, L., Greque de Morais, E., Trovão, M., Machado, A., Carvalho, B., Carneiro, M., Maia, I., Soares, M., Duarte, P., & Barros, A. (2020). Isolation and characterization of novel Chlorella vulgaris mutants with low chlorophyll and improved protein contents for food applications. *Frontiers in Bioengineering and Biotechnology*, *8*, 469.

Show, K.-Y., Lee, D.-J., Tay, J.-H., Lee, T.-M., & Chang, J.-S. (2015). Microalgal drying and cell disruption–recent advances. *Bioresource Technology*, *184*, 258-266.

Slade, R., & Bauen, A. (2013). Micro-algae cultivation for biofuels: cost, energy balance, environmental impacts and future prospects. *Biomass and bioenergy*, *53*, 29-38.

Srivastava, N., Srivastava, M., Mishra, P., Upadhyay, S., Ramteke, P. W., & Gupta, V. K. (2019). Sustainable Approaches for Biofuels Production Technologies. *Biofuel Biorefin Technol*, *7*, 121-146.

Torres-Tiji, Y., Fields, F. J., & Mayfield, S. P. (2020). Microalgae as a future food source. *Biotechnology advances*, *41*, 107536.

Van Eijck, J., Batidzirai, B., & Faaij, A. (2014). Current and future economic performance of first and second generation biofuels in developing countries. *Applied Energy*, *135*, 115-141.

Vignesh, N. S., Vimali, E., Sangeetha, R., Arumugam, M., Ashokkumar, B., Ganeshmoorthy, I., & Varalakshmi, P. (2020). Sustainable biofuel from microalgae: application of lignocellulosic wastes and bio-iron nanoparticle for biodiesel production. *Fuel*, *278*, 118326.

Yi, Z., Su, Y., Xu, M., Bergmann, A., Ingthorsson, S., Rolfsson, O., Salehi-Ashtiani, K., Brynjolfsson, S., & Fu, W. (2018). Chemical mutagenesis and fluorescence-based high-throughput screening for enhanced accumulation of carotenoids in a model marine diatom Phaeodactylum tricornutum. *Marine drugs*, *16*(8), 272.

Zarrinmehr, M. J., Daneshvar, E., Nigam, S., Gopinath, K. P., Biswas, J. K., Kwon, E. E., Wang, H., Farhadian, O., & Bhatnagar, A. (2022). The effect of solvents polarity and extraction conditions on the microalgal lipids yield, fatty acids profile, and biodiesel properties. *Bioresource Technology*, *344*, 126303.

Zhang, J., Xia, A., Chen, H., Nizami, A.-S., Huang, Y., Zhu, X., Zhu, X., & Liao, Q. (2022). Biobased carbon dots production via hydrothermal conversion of microalgae Chlorella pyrenoidosa. *Science of The Total Environment*, *839*, 156144.

Zhang, Y., Li, Y., Zhang, X., & Tan, T. (2015). Biodiesel production by direct transesterification of microalgal biomass with co-solvent. *Bioresource Technology*, *196*, 712-715.

Zhang, Y., Soldatov, S., Papachristou, I., Nazarova, N., Link, G., Frey, W., & Silve, A. (2022). Pulsed microwave pretreatment of fresh microalgae for enhanced lipid extraction. *Energy*, *248*, 123555.

Zheng, H., Yin, J., Gao, Z., Huang, H., Ji, X., & Dou, C. (2011). Disruption of Chlorella vulgaris cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. *Applied biochemistry and biotechnology*, *164*, 1215-1224.

Zinnai, A., Sanmartin, C., Taglieri, I., Andrich, G., & Venturi, F. (2016). Supercritical fluid extraction from microalgae with high content of LC-PUFAs. A case of study: Sc-CO2 oil extraction from Schizochytrium sp. *The Journal of Supercritical Fluids*, *116*, 126-131.