EVALUATION OF MIXED MICROALGAE SPECIES BIOREFINERY OF *DESMODESMUS* SP. AND *SCENEDESMUS* SP. FOR BIOPRODUCTS SYNTHESIS

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ABSTRACT

Microalgae is known to produce numerous bioactive compounds for instance proteins, fatty acid, polysaccharides, enzymes, sterols, and antioxidants. Due to their valuable biochemical composition, microalgae are regarded as a very intriguing source to produce novel food products and can be utilised to improve the nutritional content of traditional foods. Additionally, microalgae are used as animal feed and additives in the cosmetics, pharmaceutical as well as nutraceutical industries. As compared to other terrestrial plants and other microorganisms, microalgae possess few advantages: (1) rapid growth rate; (2) able to grow in non-arable land and harsh cultivation conditions; (3) low nutritional requirements; (4) high productivity; and (5) reduce emission of carbon dioxide. Despite the large number of microalgae species found in nature, only a few species are identified and commercialized such as *Chlorella* sp., *Spirulina* sp. *Haematococcus pluvialis*, *Nannochloropsis* sp. and *Chlamydomonas reinhardtii*, which is one of the major obstacles preventing the full utilisation of microalgae-based technology.

This thesis provides information on the overall composition of mixed microalgae species, *Desmodesmus* sp. and *Scenedesmus* sp., for instance protein, carbohydrate, lipid, antioxidants, and pigment. This thesis firstly introduces the application of triphasic partitioning (TPP) in the extraction and partitioning of the biomolecules from the microalgae. The latest advancement of technology has evolved from a liquid biphasic flotation (LBF) to TPP. T-butanol and ammonium sulphate are used in TPP to precipitate desired

biomolecules from the aqueous solutions with the formation of three layer. TPP is a simple, time- and cost- efficient, as well as scalable process that does not require toxic organic solvents. Lipase is abundantly produced by microbes, bacteria, fungi, yeast, mammals, and plants. Lipase is widely used in the oleochemical, detergent, dairy, leather, cosmetics, paper, cosmetics, and nutraceutical industries. Therefore, this thesis also discusses the possibility of identifying and extracting enzyme lipase from the microalgae using LBF. Several parameters (volume and concentration of solvents, weight of biomass, flotation kinetics and solvent types, etc.) have been investigated to optimize the lipase extraction from LBF.

Chlorophyll is the main pigment present in the microalgae. Thus, this work proposes the digital imaging approach to determine the chlorophyll concentration in the microalgae rapidly because the chlorophyll content has a significant impact on microalgae physiological health status as well as identifies the chlorophyll concentration in the production of by-products. Lastly, microalgae oil can be used as the feedstock for biodiesel as well as nutraceutical, pharmaceutical, and health-care products. The challenge in the lipid extraction is the co-extraction of chlorophyll into the oil, which can have serious consequences for downstream processing. Therefore, the removal of the chlorophyll from the microalgae using activated clay or sodium chlorite in the pre-treatment procedure are examined. The research achievements in these works and future opportunities are highlighted in the last chapter of the thesis.

PREFACE

CHAPTER 1 and **CHAPTER 2** focused on the introduction, problem statement, objectives, literature review and the methodology followed to achieve the objectives.

CHAPTER 3 outlines the application of triphasic partitioning technique to extract the biomolecules from the microalgae, followed by the quantification of the extracted biomolecules, such as carbohydrates, proteins, lipids, and antioxidants. The mixed microalgae species are subjected to three phase partitioning consists of ammonium sulphate and t-butanol to form three layers which are top, middle and bottom phases, that comprised of lipid, protein and carbohydrates, respectively. Protein content of the microalgae species is found to be 0.453 ± 0.003 g/L with the separation efficiency of 95.43%. The carbohydrates content in the mixed microalgae species is found to be 1.17 ± 0.04 mg/mL. The carbohydrates content is high as compared to the protein and lipid. The lipid profile obtained from gas chromatography shows that the highest peak is seen for C11 (0.1870 mg/g), followed by C16 (0.0459 mg/g), C12 (0.0053 mg/g) and lastly C20 (0.0012 mg/g). The gas chromatography analysis results reveals that the fatty acid methyl esters profiles of the mixed microalgae species contain an abundance of saturated fatty acids (undecanoic acid, lauric acid) as compared to unsaturated fatty acids (palmitoleic acid, eicosapentaenoic acid). Overall, the application of TPP as the platform for bioseparation procedures is versatile and functional.

CHAPTER 4 focuses on the implementation of RGB imaging modules using smartphone camera to predict the concentration of chlorophyll in microalgae through the incorporation of regression model and artificial neural network. Each colour component in the three colour models for various solvents type are considered as a single variable. It has been observed that the colour chromate and its chlorophyll content are linearly associated. Red-green-blue index is found to be the most important feature in forecasting chlorophyll concentration. The multilayer perceptron model with the extraction solvents is robust to predict the chlorophyll concentration of microalgae with R^2 of 0.76 as compared to the model without solvents as the input (R^2 = 0.674). The accuracy between measured and predicted chlorophyll concentration was moderate high with average relative error of 48.6 %. Multilayer perceptron model provides better accuracy (high R^2 value) for the prediction of chlorophyll concentration as compared to linear regression. Using experimental data, multilayer perceptron model can be successfully trained to interpret the relationships between the input variables and correctly predict the output. The image processing technique can be considered as a valid approach for microalgae chlorophyll prediction.

CHAPTER 5 discusses on the lipase extraction from the microalgae using liquid biphasic flotation system and the optimization parameters of the extraction process. 400 mg microalgae biomass, 99.8% ethanol and 300 g/L ammonium sulphate combination, volume ratio of 1: 0.83, pH of 7.0, air flotation of 150 cc/min and 10 min flotation time are discovered to be optimal

LBF conditions. The optimized LBF system is found to have a maximum lipase recovery of 90.4%. The recycled solvents also demonstrate satisfactory performance, indicating the possibility to reuse the solvents in the future. This could reduce the cost to produce biomolecules from the microalgae and other microorganisms as well as minimize the environmental impact.

CHAPTER 6 depicts a novel pre-processing of microalgae biomass prior to lipid extraction using sodium chlorite combined with organic solvents. The findings reveal that approximately 70% of the chlorophyll in biomass can be removed. The oil yielded by chlorophyll-reduced biomass is orange-green colour and fluidic. In the treated biomass, the proportion of the saturated fatty acids reduces whereas the unsaturated fatty acids level increases. Different treated biomass demonstrates varied lipid loss rate, with 13% being the lowest for DMSO- $NaClO_2$. The biochemical composition including carbohydrate and proteins in treated biomass do not affect significantly as compared to untreated biomass. The outcome shows the potential of $NaClO_2$ pre-treatment approach in enhancing the production of microalgae oil.

CHAPTER 7 compares the chlorophyll removal efficiency using different types of activated clay, which are kaolinite, Fuller's earth and bentonite clay. Bentonite clay is found to remove chlorophyll effectively (76.04%) as compared to kaolinite and Fuller's earth due to the presence of porous materials that facilitate adsorption. Additionally, the conditions of high temperature (79.69%) and ultrasound treatment (84.24%) demonstrate

satisfactory chlorophyll removal efficiency. Another promising finding is that chlorophyll can be adsorbed on the clays without affecting the lipid composition in the microalgae extract significantly. In a single sequential run, using spent bentonite clay can yield the outcomes comparable to those of virgin bentonite clay. The present feasibility study proves the potential of reusability of the bentonite in the removal of chlorophyll from the microalgae lipid extracts.

Lastly, **CHAPTER 8** summarizes the complete thesis by providing the challenges and scopes of the present work as well as states on the future scope of the work along with the conclusion.

LIST OF PUBLICATIONS RELEVANT TO THE THESIS

Journal Publication

1. **Doris Ying Ying Tang**, Kit Wayne Chew, Francesco G. Gentili, Chongqing Wang, Heli Siti Halimatul Munawaroh, Zengling Ma, Fubao Sun, Muthusamy Govarthanan, Sarah Alharthi, Pau Loke Show. “Dechlorophyllization of Microalgae Biomass for the Bioconversion into Lipid-Rich Bioproducts”. Industrial & Engineering Chemistry Research (2023)
2. **Doris Ying Ying Tang**, Kit Wayne Chew, Francesco G. Gentili, Tonni Agustiono Kurniawan, Young-Kwon Park, Heli Siti Halimatul Munawaroh, Saravanan Rajendran, Zengling Ma, Sarah Alharthi, Walaa F. Alsanie, Pau Loke Show. "Performance of bleaching clays in dechlorophyllisation of microalgal oil: A comparative study." Process Biochemistry 129 (2023): 94-101.
3. **Doris Ying Ying Tang**, Kit Wayne Chew, Huong-Yong Ting, Yuk-Heng Sia, Francesco G. Gentili, Young-Kwon Park, Fawzi Banat, Alvin B. Culaba, Zengling Ma, and Pau Loke Show. "Application of regression and artificial neural network analysis of Red-Green-Blue image components in prediction of chlorophyll content in microalgae." Bioresource Technology 370 (2023): 128503.
4. **Doris Ying Ying Tang**, Dinh-Toi Chu, Sakhon Ratchahat, Fawzi Banat, Kit Wayne Chew, Francesco G. Gentili, and Pau Loke Show.

"Investigation and Screening of Mixed Microalgae Species for Lipase Production and Recovery using Liquid Biphasic Flotation Approach." Journal of the Taiwan Institute of Chemical Engineers 142 (2023): 104646.

5. **Doris Ying Ying Tang**, Kit Wayne Chew, Shir Reen Chia, Huong-Yong Ting, Yuk-Heng Sia, Francesco G. Gentili, Zengling Ma, Mukesh Kumar Awasthi, and Pau Loke Show. "Triphasic partitioning of mixed Scenedesmus and Desmodesmus for nutrients' extraction and chlorophyll composition prediction for algae bloom." Environmental Technology (2022): 1-12.
6. **Doris Ying Ying Tang**, Guo Yong Yew, Apurav Krishna Koyande, Kit Wayne Chew, Dai-Viet N. Vo, and Pau Loke Show. "Green technology for the industrial production of biofuels and bioproducts from microalgae: a review." Environmental Chemistry Letters 18, no. 6 (2020): 1967-1985.
7. **Doris Ying Ying Tang**, Kuan Shiong Khoo, Kit Wayne Chew, Yang Tao, Shih-Hsin Ho, and Pau Loke Show. "Potential utilization of bioproducts from microalgae for the quality enhancement of natural products." Bioresource technology 304 (2020): 122997.
8. Vasistha, Shrasti, Anwesha Khanra, Monika Prakash Rai, Shakeel Ahmad Khan, Zengling Ma, Heli Siti Halimatul Munawaroh, **Doris Ying Ying Tang**, and Pau Loke Show. "Exploring the Pivotal Significance of

Microalgae-Derived Sustainable Lipid Production: A Critical Review of Green Bioenergy Development." Energies 16, no. 1 (2023): 531.

9. Peter, Angela Paul, Guo Yong Yew, **Doris Ying Ying Tang**, Apurav Krishna Koyande, Kit Wayne Chew, and Pau Loke Show. "Microalgae's prospects in attaining sustainable economic and environmental development." Journal of Biotechnology (2022).

Conference Presentation

1. **Doris Ying Ying Tang**, Xuefei Tan, Kit Wayne Chew, Shir Reen Chia, Huong-YongTing, Yuk-Heng Sia, Francesco G. Gentili, Pau Loke Show. Biochemical Compositions and Prediction of Chlorophyll Content in Microalgae for Potential Therapeutic Applications in 6th Postgraduate Colloquium for Environmental Research, Resort World Langkawi, Kedah, Malaysia (9 - 11 June 2022)
2. **Doris Ying Ying Tang**, Xuefei Tan, Kit Wayne Chew, Shir Reen Chia, Huong-Yong Ting, Yuk-Heng Sia, Francesco G. Gentili, Pau Loke Show. Biochemical Compositions and Prediction of Chlorophyll Content in Microalgae for Potential Therapeutic Applications in 5th Rencontres de Quy Nhon: International Biology Conference 2022, ICISE, Quy Nhon, Binh Dinh, Vietnam (17-19 August 2022)
3. **Doris Ying Ying Tang.** Pigment Extraction from Microalgae using Integrated Ultrasound-assisted Liquid Biphasic Flotation in ESS-JSS-AOSS 1st Joint Sonochemistry Conference (8-10 Nov 2021)

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acetyl-CoA	Acetyl coenzyme A
ALA	Alpha-linolenic acid
ATPS	Aqueous two-phase system
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeat
CMYK	Cyan, Magenta, Yellow and Black
DHA	Docosahexaenoic acid
DME	Dimethyl ether
EBA	N-ethylbutyl amine
DNA	Deoxyribonucleic acid
EPA	Eicosapentaenoic acid
GC-FID	Gas chromatography with flame ionisation detector
GUI	Graphical User Interface
HSL	Hue, saturation and luminance
LBF	Liquid biphasic flotation

LBS	Liquid biphasic system
LDL	Low-density lipoprotein
LR	Linear regression
MLP	Multilayer perceptron
MTBE	Methyl-tert-butyl
MUFA	Monounsaturated fatty acids
PCB	Polychlorinated biphenyls
PUFA	Polyunsaturated fatty acid
RGB	Red, Green, and Blue
RNAi	Ribonucleic acid interference
SDNs	Site-directed nucleases
SFAs	Saturated fatty acids
SFE	Supercritical fluid extraction
SEs	Steryl esters
TAG	Triacylglycerols
TALENs	Transcription activator-like effector nuclease

TEAC	Trolox equivalent antioxidant capacity assay
TPC	Total phenolic content
TPP	Triphasic partitioning
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
WEs	Wax esters
ZFNs	Zinc-finger nucleases

CHAPTER 1 INTRODUCTION

1.1 Research background

Microalgae are a group of single-celled organisms or also referred to as phytoplankton (i.e., 'phyto'=plant, 'planktos'= made to wander) which can be categorised by various morphological traits [1]. Various scientific studies had showed the great potential of microalgae as a reliable and sustainable feedstock for production of biofuel and diverse valuable bioresources such as polysaccharides, lipids, proteins, enzymes, vitamins and carotenoids that can be commercialized in different industries [2, 3]. Approximately 7,000 tons of dry algal biomass are produced worldwide annually and the global market for algae biomass is worth between USD 3.8 to 5.4 billion [4]. This statistic shows that the microalgae industry is gaining the popularity worldwide and can be utilised extensively through the incorporation into industrial products in different sectors in the future. Nowadays, there are emerging some microalgae-based bioproducts, especially derived from *Chlorella* sp. and *Spirulina* sp., on the market from different industry verticals, such as food and beverages, pharmaceutical nutraceutical, and dietary supplements sectors. However, microalgae are underutilized and needed high attention from the researchers worldwide to cope with the high demands from increased population growth. There are some features possessed by microalgae that enable it to be exploited widely such as fast growth rate, simple cultivation requirements (i.e., require water, sunlight, carbon dioxide and the ability to survive in harsh conditions. In summary, the first step in assessing the

potential of microalgae in various industries is to determine its biochemical composition.

One of the examples is that microalgae known to produce polyunsaturated fatty acids, Omega-3 fatty acids. The main omega-3s are eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA). Omega-3 fatty acids shown to reduce cardiovascular risks in decreasing the cholesterol level, reduce blood pressure and prevent the risk of atherosclerosis. Recently, the omega-3 oil supplements market has been gaining notable momentum because the people nowadays more concern about their health by taking supplements to boost their immune system and prolong the life. In the past, omega-3 fatty acids are extracted from the marine sources but facing the issues of marine pollution, decrease global fish stock market and not suitable for vegetarian consumption. Therefore, microalgae biomass which contain high proportion of fatty acids hold the promise to become replacement of marine omega-3 sources, as there have some microalgae omega-3 capsule being sell in the market [5].

In addition, there have literature studies that microalgae also capable of synthesis enzymes, such as lipase [4, 6-8]. Lipases (EC 3.1.1.3) is the enzyme that perform the hydrolysis of triacylglycerols (TAG). Lipases are commonly used in the immobilized form in pharmaceutical industries. The most organisms that used to synthesis lipase in the commercial scale is *Candida antarctica* (Lipase B), CAL-B. CAL-B is widely used as the catalyst in the pharmaceutical reaction in the drug development process, for example

methotrexate (anticancer and anti-rheumatic drug) and azelaic acid derivatives [9]. Previously, the lipases are commonly produced from the bacteria, fungus, plant, or animals. The microalgal lipases are less studied as compared to the microbial lipases. Demir and Tükel [6] first characterised the purified lipase from *Spirulina platensis*. This monomeric protein was specific for 3-positon in the ester bond and had the molecular weight of 45 kDa. In another years, Savvidou, Sotiroudis [10] isolated the lipases from *Nannochloropsis oceanica* that showed the hydrolysis ability against the *p*NP esters of fatty acids and optimum pH of 7. Yong, Lim [7] also characterised the lipase from *Botryococcus sudeticus* that can be active in wide ranges of temperature and pH as well as has the potential to be explored for industrial purposes. The lipases shown in these literature studies demonstrated good thermal stability, so has the potential to be applied in different industries. In industrial application, lipase commonly produced by the microorganisms such as bacteria and fungus. However, the cost of these enzymes are high [11], increasing the production cost. Therefore, microalgae have emerged as the promising candidate to produce and replace the role of microbial in producing these enzymes. Through these literature studies, microalgae had shown the capability to produce various enzymes, including the lipase.

Chlorophyll is the main pigment in the microalgae which give the microalgae extracts their distinctive green colour. However, the presence of chlorophyll during the extraction of lipids and other biomolecules from microalgae can cause rancidity and oxidation issues. Additionally, the

greenish colour of the lipid-based microalgae products makes it unattractive for product development and undesired for consumers, lowering their purchasing power. Therefore, the removal of chlorophyll is a vital stage in the usage of microalgae biomolecules for commercial purposes. The chlorophyll can be removed by the adsorbents such as activated clays and carbon as well as the pre-processing of microalgae biomass via bleaching agent or saponification reaction. During the chlorophyll removal, it is essential to ensure that there are non-significant fatty acid losses. The results can shed fresh light on how to prepare microalgae extracts in an environmentally friendly manner without having a problem with discolouration.

1.2 Problem statement

The biochemical composition (carbohydrates, proteins, and lipids) of the mixed microalgae species, *Desmodesmus* sp. and *Scenedesmus* sp. have not been studied and characterized yet. These biomolecules can be exploited for the use in different industries in the future. Besides, the literature study shows the great capacity of microalgae cells to synthesize enzymes , although there is no industrial production of enzymes from microalgae [4], especially lipases. These enzymes can be found in a diverse range of species such as animals, plants, bacteria, yeasts and fungi [12, 13]. Owing to the plethora of their hydrolytic and synthetic activities, lipases have received considerable attention with regard to industrial applications in the fields of food technology, detergents, biofuels, compound synthesis, chemical industry and biomedical sciences [14]. However, to date, not more than about

20 lipases have been industrially exploited [15]. Despite the enormous amount of published data on plant, animal and microbial derived lipolytic enzymes, little information is available on microalgal lipases [6, 16].

The conventional method to measure the chlorophyll concentration of microalgae is via spectrophotometric method. This method requires sample destruction through few physical and chemical extraction procedures and takes place in a laboratory. This method is labour-intensive and time-consuming. Therefore, digital imaging approach using colour imaging systems (Red-Green-Blue, RGB) is proposed. This approach is inexpensive and can be applied using smartphones. The prediction of chlorophyll concentration in the microalgae is conducted using linear regression model and artificial neural network. The question of whether an RGB-based system can achieve a similar result to spectrophotometry method in a specific absorbance in the determination of chlorophyll concentration has become a research question.

Despite the high potentiality of microalgal biomass to produce various biocompounds, such as carbohydrates, proteins and lipids, there are many technological and economical challenges impeded the commercialization of microalgae-based products by considering the cost effectiveness and sustainability issue. Currently, the production of microalgae lipid does not meet the economic aspects yet due to the presence of chlorophyll. During the lipid extraction from microalgae biomass, the liposoluble intracellular pigments such as chlorophyll will be co-extracted into crude oil and thus

affecting oil quality and limit its large-scale application. Chlorophyll is insoluble in water but readily dissolved in organic solvents including ethanol, acetone, and chloroform [17] and these solvents are used commonly for lipid extraction. To date, the studies regarding the removal of chlorophyll from microalgae oil and the developed methods were inefficient. Chen, Liu [18] used bleaching method to remove the chlorophyll and carotenoids in *Scenedesmus* sp. and this method was successful reduce the pigments contents. However, this method was not useful for large-scale application and the bleaching agents used was not suitable for the production of food, nutraceuticals and pharmaceuticals products. Li, Xu [19] removed the chlorophyll in microalgae, *Scenedesmus* sp. biomass via saponification reaction, followed by the oil extraction. The end product was transparent orange *Scenedesmus* sp. oil with 96% removal rate of chlorophyll in biomass. However, the pigments composition of extracted oil became carotenoids-based and the concern of the feasibility of this method with other microalgae species and other cultivation stages was raised. Hence, an effective chlorophyll removal technique is needed to produce the high quality and pure microalgae oil.

1.3 Research objectives

This study focuses on identification and quantification of the biomolecules from the mixed microalgae species, *Desmodesmus* sp. and *Scenedesmus* sp. as there have less studies done on it. Besides, dechlorophyllization process is required to produce high-quality microalgae by-products.

Furthermore, the benefits of RGB imaging over spectrophotometry method is rapid and can be applied in field conditions.

Considering above, the objectives of the research are:

a) To assess and characterize the biochemical composition (lipids, carbohydrates and proteins) in mixed microalgae species
b) To determine the presence of lipase in the microalgae and optimize the extraction of enzyme lipase using liquid biphasic flotation system
c) To estimate and predict the chlorophyll concentration using statistical model, for example regression and artificial neural network models with the incorporation of colour index
d) To compare the chlorophyll removal efficiency in the microalgae lipid using different types of clay powders
e) To evaluate the performance of bleaching agent, sodium chlorite in the pre-processing of microalgae biomass as the dechlorophyllization process

1.4 Research contributions

The research scopes proposed in Section 1.3 were achieved, and each scope is described in detail in Chapter 3 through Chapter 7. The following is a summary of the research contributions of this thesis:

a) **Triphasic partitioning of biomolecules from the microalgae:** The biochemical composition of mixed microalgae species, *Desmodesmus* sp. and *Scenedesmus* sp. was assessed using TPP system to form

three layers, top, middle and bottom phases, that comprised of lipid, protein and carbohydrates, respectively. The biochemical characterization was then conducted. The bottom phase (carbohydrate) was tested using phenol-sulphuric acid method. For the middle phase, Thermo Scientific™ Pierce™ Bicinchoninic Acid Protein Assay Kit was used to estimate the protein concentration with bovine serum albumin as the standard. The top phase was determined quantitatively and qualitatively using gravimetric method and gas chromatography with flame ionisation detector (GC-FID). The antioxidant activity in the crude microalgae extract was evaluated using total phenolic content (TPC) assay and Trolox equivalent antioxidant capacity assay (TEAC).

b) **Extraction of lipase enzyme using liquid biphasic flotation:** The lipase was extracted using liquid biphasic flotation as the protein homogenates from the extracts was found to contain lipase activity. The lipase activity was evaluated using colorimetric method with p-nitrophenyl palmitate as the substrate. The extraction parameters, such as extraction time, mass of microalgae biomass as well as the type, volume and concentration of salt and alcohol were evaluated and optimized.

c) **RGB in digital image processing to estimate the microalgae chlorophyll concentration:** A rapid and non-invasive method based on colorimetry of digital images to predict microalgae chlorophyll

concentration is introduced. This work pioneers the use of smartphone camera for image acquisition. The chlorophyll concentration can be estimated by analysing the spectral information of each colour component. Next, this study aimed to select a suitable extraction solvent (acetone, methanol, ethanol and dimethyl sulfoxide) to extract chlorophyll as an active ingredient for their potential application in various sectors.

d) **Pre-processing or pre-treatment of microalgae biomass in the chlorophyll removal:** This present study proposed the use of novel pre-processing of microalgae biomass using sodium chlorite combined with organic solvents (methanol, ethanol, acetone, dimethyl sulfoxide). The free fatty acid, chlorophyll, carotenoids, and oil retention levels were measured. The chlorophyll removal efficiency from crude microalgae oil was compared with bleaching methods such as activated carbon and clay as well as the saponification methods using sodium hydroxide.

e) **Activated clay as dechlorophyllization procedure:** Three different clay types are used to assess and compare their dechlorophyllization ability of microalgae oil. The clays involved are kalininite, Fuller's earth and bentonite clay. Ratio of microalgae and clay powder, temperature, contact time and the combination of ultrasonic treatment were optimized to evaluate the removal efficiency of chlorophyll. Clay

powders were characterized using scanning electron microscopy with energy dispersive X-ray spectroscopy.

1.5 Outline of Thesis

The outline of thesis consists of eight (8) chapters and stated as followed. **CHAPTER 1** covers the research backgrounds, research objectives, contribution of this research and the current problem statement faced in the downstream processing of biomolecules from microalgae. **CHAPTER 2** covers the literature reviews on the introduction of microalgae species involved in this study, biochemical composition of microalgae, potential market of microalgae products as well as liquid biphasic system and its advancement, triphasic partitioning technologies. **CHAPTER 3** outlines the extraction using triphasic partitioning and quantify the biomolecules (carbohydrates, proteins, lipids and antioxidants) in the microalgae biomass. **CHAPTER 4** illustrates the RGB imaging approach using smartphone camera incorporating with the regression model and artificial neural network in the prediction of chlorophyll concentration in microalgae. **CHAPTER 5** reports the lipase extraction using liquid biphasic flotation system and the optimization parameters of the process. **CHAPTER 6** depicts a novel pre-processing of microalgae biomass prior to lipid extraction using sodium chlorite combined with organic solvents. **CHAPTER 7** compares the chlorophyll removal efficiency using different types of activated clay. Lastly, in **CHAPTER 8** summarises the list of research accomplishments and the future research recommendations for the research gaps.

1.5.1 Research summary

This thesis considers the downstream processing of microalgae as the main subject of its study. In **Chapter 3**, the biochemical composition of microalgae cultures consisted of *Desmodesmus* sp. and *Scenedesmus* sp. was assessed using TPP. The results highlighted that the biomass consisted of varied concentration of carbohydrates, protein, and lipids. Phenolic compounds and antioxidant activity were high, at 60.22 mg/L of phenolic compounds and 90.69% scavenging activity respectively. In the past decades, spectrophotometric assay had been used to determine chlorophyll concentration. The key problems with this technique were requirement of sample destruction and time consuming if involved large number of samples. Therefore, RGB analysis incorporated with linear regression and artificial neural network models were proposed in **Chapter 4** to predict the chlorophyll concentration using smartphone camera. Our results demonstrated that in comparison to CMYK model, RGB model offers a better correlation with high R^2. The Green in RGB index was the most promising way to estimate chlorophyll concentration in microalgae. The predicted chlorophyll concentration by the regression and multilayer perceptron models and measured chlorophyll concentration by standard laboratory procedure are found to be significantly correlated. By comparing extraction solvents, acetone showed the strongest correlation and was a suitable choice for extracting chlorophyll.

Aside from the assessment of few primary biomolecules in the microalgae, less literature studies to date had examined the presence of lipases in the microalgae as demonstrated in **Chapter 5**. The lipase was extracted using liquid biphasic flotation as the protein homogenates from the extracts was found to contain lipase activity. The result showed that the lipase production was optimized at 225 mg of microalgae biomass, 15 min flotation time with 125 cc/min as well as 99.8% ethanol and 275 g/L ammonium sulphate with volume ratio of 1: 1. Lipase recovery yield of optimal LBF system was 70.3% with separation efficiency of 82.0% and purification factor of 7.45.

Despite the fact that chlorophyll exhibited various health benefits, but the presence of chlorophyll during the lipid extraction will affect the quality and storage stability of microalgae by-products. Therefore, microalgae were dechlorophyllized using different methods, such as pre-treatment with sodium chlorite (**Chapter 6**) and activated clay powder (**Chapter 7**). The results in **Chapter 6** suggested that approximately 70% of the chlorophyll in biomass was removed. The oil yielded by chlorophyll-reduced biomass was orange-green colour. The proportion of fatty acids of treated biomass decreased to different extent. The performance of chlorophyll removal and lipid loss rate demonstrated by DMSO-sodium chlorite was satisfactory. The pigment composition shifted toward carotenoids. The biochemical composition in treated biomass did not affect significantly as compared to untreated biomass. Lastly, another promising finding in **Chapter 7** showed that

chlorophyll can be adsorbed on the clays without affecting the lipid composition in the microalgae extract significantly. Bentonite clay is found to remove chlorophyll effectively (76.04%) than kalininite and Fuller's earth. FESEM result reveals the presence of porous materials that facilitate adsorption. Additionally, the conditions of high temperature (79.69%) and ultrasound treatment (84.24%) demonstrate satisfactory removal efficiency of chlorophyll. In summary, these findings demonstrated the potential of microalgae as a substitute of the natural source of several significant compounds in various sectors. Additional research on this strain was required to generate microalgae-based products that would be more diverse and economically competitive.

1.6 Overview of the Research Process

The flow chart of illustrating the scopes in this research is shown in **Figure 1.1. Overview of the research project.**. A simple and efficient technique known as TPP, was used to extract biomolecules from the microalgae. LBF has been effectively applied for microalgae lipase extraction. The system parameters such as biomass weight, solvent types, concentration, volume and time were varied to achieve optimum results. The microalgae were also subjected to various chlorophyll removal techniques. Advanced technology such as digital imaging was integrated to determine the chlorophyll concentration using smartphone camera.

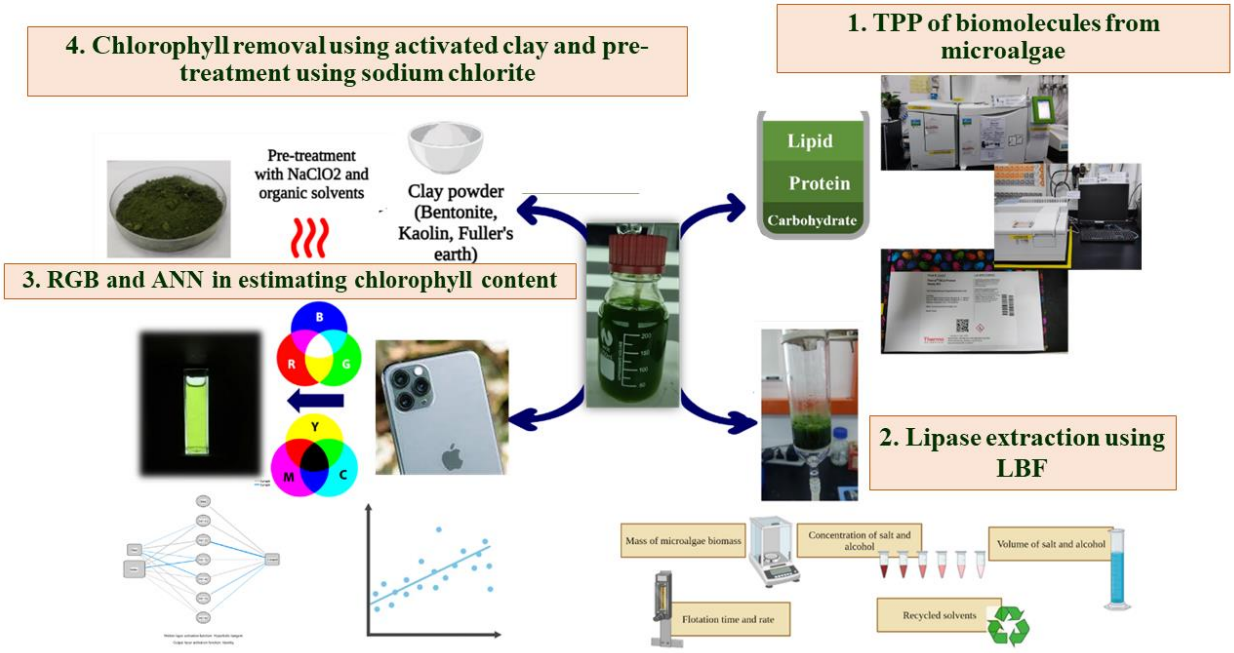


Figure 1.1. Overview of the research project.

CHAPTER 2 LITERATURE REVIEW

2.1 Microalgae

Microalgae are microscopic organisms that contain chlorophyll and are found in freshwater and marine habitats [20]. The term “microalgae” refers to prokaryotic cyanobacteria and eukaryotic photosynthetic microorganisms primarily found within the taxa *Chlorophyta*, *Rhodophyta*, *Glaucocystophyta*, *Euglenophyta*, *Chlorarachniophyta*, *Heterokonta*, *Haptophyta*, *Cryptophyta* and *Alveolata* [21]. The potential uses of microalgae extend beyond the products studies to date [4]. Recent studies emphasizes the potential of algal biomass as an alternative to fossil fuels as raw material for the production of biofuels such as biodiesel, bioethanol, biohydrogen and biomethane [22, 23]. Several reports have established that microalgae can be sources of biomass for the production of fine chemicals production including chlorophylls, β-carotene , astaxanthin [24, 25], phycocyanin and omega-3 fatty acids. There are also numerous potential commercial applications of microalgae, including in the paper industry as fiber/polymer composites and as a filler material [26, 27], in cosmetics formulations [28], for soil restoration, as supplements in animal feed [29], for phycoremediation [30, 31] and in therapeutic supplements for their polysaccharides (β-glucan) [20], essential amino acids , lipids (tri- and diglycerides, phospholipids, glycolipids), alkenes, alginates (from brown algae), agar and carragenates (from red algae) [32]. Additionally, microalgae are reported to efficiently adsorb and recover heavy metals [33] and can be used in wastewater treatment in integrated processes [34]. In

short, the microalgae are able to synthesize various essential compounds to be used in different industries. In this project, the biochemical composition of the mixed microalgae species is assessed, evaluated and characterized. The microalgae species involved are *Desmodesmus* sp. and *Scenedesmus* sp. because the literature studies conducted on these two microalgae species are inadequate.

2.1.1 *Desmodesmus* sp.

Desmodesmus sp. is categorised as the freshwater green microalgae. *Desmodesmus* sp. are round-shaped, non-motile, single-celled coccoid microalgae lacking flagellae and rigid shells. Their cells possessed diverse epistructures on their cell surfaces: warts, spines (bundle of tubes of different length) or single elongated tubes, and rosettes. The rupture line was visible without accompanying warts. The cell wall structure of the *Desmodesmus* sp. consisted of a thick inner polysaccharide (cellulose- and/or hemicellulose-containing) layer and a thin outer sporopolleninic layer. The inner layer varied in thickness and osmiophilicity and was comprised by fine granules and short microfibrils [35].

Figure 2.1. *Desmodesmus* sp. (Taken from [36], reprinted under the terms of the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited)

2.1.2 *Scenedesmus* sp.

Scenedesmus sp. are common freshwater green algae and usually used as pollution indicator where they can adapt and grow well in polluted water or sewage. The genus *Scenedesmus* is characterized by the following morphological features: flat or slightly curved coenobia which are composed of 2-32 cells that are linearly or laterally arranged in 1 or 2 rows and usually surrounded by mucilage. The cells elongate or cylindrical, ovoid, irregular, dactylococcoid, ellipsoid to ovoid with apices usually rounded. Some *Scenedesmus* sp. strains appeared as spiny and changed its morphology when exposed to biochemical released by grazer at which the presence of grazer will induce formation of coenobia to increase its overall size, to reduce vulnerability of cells against grazing. The chloroplast is parietal and usually with a single pyrenoid [37]. Asexual reproduction usually occurs with autospores which are released by fracture of lateral cell walls [38].

Figure 2.2. *Scenedesmus* sp. (Taken from [39], Reprinted under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited)

2.2 Potential or advantages of microalgae for the production of high-value compounds

Microalgae biomass offers many benefits by utilising the bioactive compounds to generate promising and sustainable source of biofuels and bioproducts. Under favourable growing conditions, microalgae are able to divide their cells within 3 to 4 hours [40]. This fast reproduction ability allows the production of biomass within 3.5 hours during exponential growth [41], allowing the biocompounds to be accumulated in a short period of time. The compounds associated with microalgae are generally recognized as safe and environmentally friendly which are beneficial and harmless for direct human consumption [42].

Microalgae cultivation does not have any significant negative impact on food security or food chains as the cultivation process does not interfere with the use of arable or fertile land that are used for conventional agriculture [43]. This is because microalgae are able to survive in harsh environmental conditions for long-term, such as wastewater environment, salt water, ice or hot springs [44-46]. Furthermore, microalgae can tolerate a broad range of pH, salinity, temperature and contaminants. Consequently, microalgae have the attainable to grow in the environments that contain excessive CO_2, sulphur oxide and nitrogen oxide [3].

Moreover, microalgae have low demands for nutritional and spatial cultivation requirements. The cultivation of microalgae requires small supply of nutrients such as light, carbon sources, CO_2, nitrogen, phosphorus and potassium [43] without the use of fertilizers like pesticides or herbicides. The manipulation of the cultivation conditions offers the possibility to enhance or induce the production and accumulation of compounds in the microalgae [47]. Nonetheless, microalgae help to reduce the greenhouse effect and global warming due to the requirement of CO_2 as a carbon source in the cultivation process and reduces the release of excess CO_2 to the environment.

2.3 Biochemical composition of microalgae

Microalgae has produced various essential biocompounds in different proportion that have high demand in food, aquaculture and animal feed, wastewater management, transportation, nutraceutical, pharmaceutical, cosmetics and personal care industries. In the aquaculture sector, microalgae biomass used for feeding larvae can reach the market prices exceeding 100 €/kg but the global production capacity of microalgae biomass is only approximately 20-30 kt annually [48].Some examples of microalgae-produced bioactive compounds including lipids, peptides or amino acids, antioxidants, polysaccharides and pigments [49].

2.3.1 Lipids

Microalgae are the unicellular microorganisms which have shown to produce lipid which can be utilised as biofuel, drugs, food or nutraceuticals. The lipid productivity in microalgae is high as compared to the traditional oil-bearing

crops, for instance corn, soybean and palm tree, and can be up to 85% for oil-producing microalgae [50, 51]. Due to the high lipid productivity, microalgae able to synthesize 58,700 litres oil per hectare [52], showing the potential as an alternative sources for biofuel or other lipid-based bioproducts. The lipid yield by the microalgae affected by the strain species, surrounding environment and cultivation conditions [53]. There are various microalgae species at which their biomass contain high lipid contents, ranged from 20% to 80% of dry biomass or dry cell weight (DCW), such as *Botryococcus braunii* (25-80%), *Dunaliella tertiolecta* (35.6%), *Chlamydomonas reinhardtii* (21%), *Chlorella vulgaris* (14-22%), *Chlorella protothecoides* (57.9%), *Nannochloropsis* sp. (37-60%), *Isochrysis* sp. (25-33%), *Neochloris oleoabundans* (35-54%), *Nannochloris* sp. (30-50%), *Schizochytrium* sp. (50-77%) and *Crypthecodinium conhi* (20%) [52, 54-56]. The microalgae lipids can be categorized into two major groups which are polar lipids (phospholipids, glycolipids) and non-polar lipids or neutral lipids (sterols and free fatty acids). Generally, the polar lipids constitute 41 to 92% of total lipids whereas non-polar lipids comprise of 5% to 51% in the microalgae [57].

Polyunsaturated fatty acid (PUFA) produced by microalgae exhibits antioxidant, antibacterial, antiviral, detoxifying capacities, prevents hypercholesterolemia, improves brain function and proved to have good immune stimulatory effects [58, 59]. Food and Drug Administration (FDA) has approved the use of DHA algal oil as an additive for infant formula by Martek Biosciences. This DHA-rich algal oil is produced from the microalgae,

Schizochytrium sp. and *Crypthecodinium cohnii*. The two main types of microalgal PUFA, DHA and EPA, are the primary source of omega-3 fatty acids with the market price around $80 – 160/kg and have the estimated market of $898.7 million at 2025 [60]. In the past few years, marine fish was the main source of DHA and EPA, however some of the issues had been arise such as not suitable for the consumption by the vegan and the unpleasant smelly produced from fish oil is not suitable to be used as a food ingredient. Moreover, the leakage of fuel oil from the ships as well as the discharge of waste chemicals comprised of mercury, dioxins, chlorinated pesticides and polychlorinated biphenyls (PCB) from the factory have resulted in the depletion of the world's fish stock.

Microalgae lipids consist of glycolipid and phospholipid as the common polar lipid is functioning as the bridge compounds between the non-polar carotenoid and polar protein. Polar lipid or structural lipid assists build-up membrane structure commonly found in the cellular wall membrane from the microalgae biomass. Apart from maintaining the cell structure, polar lipids that consist of long chains fatty acids can undergo a series of metabolic reaction to produce PUFA, for instance EPA and DHA. Generally, the majority of polar lipid found as phospholipids at which a glycerol act as backbone and phosphate as the polar molecule that attached with fatty acids [61]. The interaction of the polar lipid with cellular membrane have influenced a series of the signal processes and enzymatic functions of the cells [62]. Phospholipids have beneficial potential for human body health especially in

the prevention of coronary heart disease and cell mutation. Phospholipid also have the ability to deliver fatty acids effectively to the membrane as the immune compounds. In the other hands, glycolipids are mostly found in eukaryotic cell. The molecules of glycolipid can perform cell surface recognition with amphipathic nature to bind physically to antibody, pathogen, toxins and intact cell. This assists the discovery of smaller molecules of the affinities and specification through thin layer chromatographic and surface plasmon resonance [63]. Glycolipid have two classes which are glycolipids and glycosphingolipids, where glycolipid found in plants while glycosphingolipid is commonly found in animals.

TAG, steryl esters (SEs), and wax esters (WEs) are among the group of neutral lipids which are without charge in the molecular structure. These neutral lipid particles have the hydrophobic core which are often surrounded by the phospholipid monolayer and mobilized by lipases with hydrolases product for membrane formation [64]. Neutral lipids, especially TAG are normally used for the energy storage or as the components of lipid droplets [57] to ensure the adequate supply of metabolite energy and molecules of energy storage for the cell. Sterols or also known as phytosterols are also one of the important components of the microalgae that control the fluidity and permeability of membranes. Physterols produced by microalgae shown to exhibit cholesterol-lowering, anti-inflammatory, anticancer, antioxidant, antibacterial and antidiabetic activities [65]. In addition, fatty acids are defined as the straight or branched long aliphatic chains of carboxylic acids which can

be in saturated or unsaturated forms, for example, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and PUFA. SFA has an essential role in deciding the fuel properties. On the contrary, omega-3 or omega-6 fatty acids are the examples of PUFA and have been explored widely as the nutraceuticals due to beneficial health properties. PUFA are essential to human health to maintain brain function and cell growth. PUFA lowered the LDL (low-density lipoprotein) cholesterol (bad cholesterol), triglycerides, the build-up of plaque in the arteries and thus reduce the risk for cardiovascular disease. PUFA also helps to reduce the risk for diabetes by controlling the blood sugar. However, our bodies do not synthesise these essential fatty acids and must get from the food or supplements. In short, the composition of lipids produced is varied among the microalgae species and different lipid classes have various health and fuel properties.

2.3.2 Enzymes

Microalgae has also been reported to have great capacity to synthesize enzymes for different industrial applications such as phytases, *α*-galactosidase, protease, laccases, lipase, cellulases, amylolytic enzymes, antioxidant enzymes and carbonic anhydrase [4]. Enzymes from microorganisms could be used as catalysts in diverse industrial processes. the search for new sources of microbial enzymes is ongoing and requires sustainable solutions [66]. In 2014, the global market for industrial enzymes was estimated at USD 4.2 billion and is expected to reach nearly USD 7.1 billion by 2018 [67].

2.3.2.1 Lipase

Lipases are ubiquitous esterase enzymes belonging to class 3 hydrolytic enzymes and further assigned to subclass and sub-class 1 and 1.4 respectively (EC 3.1.1.3) [16]. Lipases are produced by animals, plants and microorganisms. They catalyse the reversible hydrolysis and synthesis of lipids [68]. Recently lipases have gained great commercial interest as they have several appealing characteristics such as not requiring cofactors, active in organic interfaces and accommodating to a broad range of substrates [69]. However, there are only a few commercially available lipases from fungi or bacterial sources that meet ideal enzyme characteristics. In 2009, characterisation of lipase from the blue-green algae, *Spirulina platensis* was reported [6]. *S*. *platensis* is a high protein, edible, filamentous cyanobacterium. According to the study, lipase isolated from *S*. *platensis* has a molecular weight of 45 kDa and an isoelectric point of 5.9. In addition, the cyanobacteria lipase showed optimal hydrolytic activity at 45 °C and pH 6.5. Godet, Hérault [70] isolated a new gene from the microalgae *Isochrysis galbana* that encode a 49 kDa lipase of 457 amino acids. The deduced protein shares similarities with known lipases. The study by Yong, Lim [7] use *Botryococcus sudeticus* UTEX 2629 as the source of extracellular lipase production. The enzyme has a molecular mass of 120 kDa, good thermal stability and its protein sequence is 90% similarity to *S*. *mobaraensis* esterase. The lipase has an optimum temperature of 50 °C and an optimum pH of 10. Lipases activities at these optimum conditions were over 2-fold and

7-fold higher compared to their lowest activities respectively, showing its potential to be applied in different industries. Savvidou, Sotiroudis [10] found a thermostable lipolytic enzyme from *Nannochloropsis oceanica*, microalgae grown in seawater that show 2.5-fold stimulation after heat treatment at 100 °C for 60 min and optimum pH of pH 7.0.

2.3.3 Polysaccharides

Polysaccharides are the essential products produced by the microalgae and the amount produced is varied among the species and genera. Many literature studies have shown that the potential of microalgae as polysaccharides producers were under investigation and had small or limited market [2]. Polysaccharides obtained from the microalgae can be used as metal ion chelators and protect against plant pathogens [71]. Algal polysaccharides contain sulphate esters that are known as sulphated polysaccharides and mainly produced by *C. vulgaris* and *S. quadricauda*. Sulphated polysaccharides comprised of ulvan, carrageenan and fucoidan. Apart from being widely used as food additives, carrageenan also demonstrated antiviral and antioxidant activities [72]. Recent work has showed that ulvan exhibited medicinal properties, for example, anticancer, antihyperlipidemic, antifungal, hepatoprotective, antiprotozoal, anti-inflammatory, antioxidant, anticoagulant, immunomodulation and enhanced skin tissue regeneration [73].

2.3.4 Proteins and Amino acid

Microalgae biomass are well known for its high protein composition such as glycoprotein from *C. vulgaris*, C-phycocyanin from *S. platensis* and phycoerthrin from *P. cruentum* [74]. Besides, microalgae such as *C. sorokiniana*, *C. minutissima*, *C. luteoviridis*, *C. sphaerica*, *A. spiralis*, *C. nivalis*, *Aphanizomenon flos-aquae*, *Scenedesmus* sp. and *Stichococcus* sp. produce mycosporine-like amino acids that are the strong ultraviolet absorbing compounds in nature [74, 75]. Microalgae has also been reported to have great capacity to synthesize enzymes for different industrial applications such as phytases, *α*-galactosidase, protease, laccases, lipase, cellulases, amylolytic enzymes, antioxidant enzymes and carbonic anhydrase [4].

Phycobiliproteins, a group of light-harvesting protein-pigment complexes that comprised of C-phycocyanin, allophycocyanin and phycoerythrin [76]. Due to the features of highly fluorescent, phycobiliproteins are used as fluorescent promising labelling reagents that can be employed in flow cytometry, fluorescence immunoassay, immunohistochemistry and other biomedical science activities [3, 43]. *Spirulina platensis* are an excellent source of phycobiliproteins, particularly c-phycocyanin, a natural blue pigment [77]. These compounds have been commercialised as the natural colourants in food industry and nutraceutical products, such as use of phycocyanin from *Spirulina* as colouring agent in soda ice cream and soda pop. The global market was valued at $60 million in 2019 with Algapharma

BiotechCorp, Columbia Bioscience and QuantaPhy. Inc are the manufacturers and suppliers of phycobiliproteins [60].

In short, the microalgae biomass has shown to produce various beneficial compounds such as carbohydrates, lipids, proteins and antioxidants which can be used to produce food, beverage, pharmaceutical, nutraceutical and biofuels. However, the challenges associated with the commercialization of microalgae-based products, for instance high production cost and low production amount, need to be identified and overcome.

2.3.5 Antioxidants and pigments

Microalgae can synthesize large quantities of carotenoids that can act as colourant and food supplements such as β-carotene, astaxanthin, canthaxanthin and phycobiliproteins. Microalgae have become an attractive option to be exploited in industrial areas as a source of natural colourant and supplement due to the emerging of health safety issues caused by synthetic colourants and supplements such as allergic reaction and hyperactivity in human [25]. The carotenoids from microalgae also possess some beneficial medical properties such as antioxidant activity that are absent in synthetic colourants [78].

Astaxanthin is a bright red secondary carotenoid, a ketocarotenoid that is known as ‘super antioxidant’ because its antioxidant activity is stronger than other carotenoids such as lutein, beta-carotene and canthaxanthin [25, 60]. Astaxanthin possess singlet molecular oxygen-quenching activity,

prevents the ultraviolet radiation, inhibits lipid peroxidation, scavenges free radicals [79] as well as exhibits anti-inflammatory and anti-apoptotic properties [80]. Astaxanthin is a promising carotenoid that has already achieved a profit of $200 million per year with an estimated market of $40 million at 2019 [60, 81]. The main microalgal producers of astaxanthin are *H. pluvialis*, *C. zofingiensis*, *C. nivalis*, *B. braunii*, *C. vulgaris*, *C. striolata*, *Monoraphidium* sp., *Chlamydocapsa* sp., *Neospongiococcum* sp., *Chlorococcum* sp. and *S. obliqus* [24, 74]. Most common researches reported that the accumulated astaxanthin in *H. pluvialis* can reach up to 3.8 – 5.0% (w/w), which depends on its cultivation condition [24]. Algatechnologies Ltd., AstaReal Inc., Beijing Gingko Group (BGG), Cyanotech Corporation and Parry's Pharmaceuticals are the major commercial producers of *Haematococcus* astaxanthin supplements [82].

Beta-carotene (β-carotene) produced naturally from *D. salina* with the market price ranged from $300 – $500/kg. At the same time, its market is forecast to reach $532 million in 2019 [83]. β-carotene is the precursor of vitamin A or also known as retinol at which the human body converts the β-carotene into vitamin A that is essential for pregnant women and children. It is found that β-carotene provides various health benefits such as protect the cornea, anti-aging, anti-cancer, immune modulation and prevent cardiovascular diseases [2]. Vitamin A deficiency will result in immune impairment, night blindness, xerophthalmia and blindness in children. In conjunction with that, it is a major public health problem in low-income

countries which mostly affect young children and pregnant women. Besides, β-carotene is also used as animal feed additives and colourant for food and beverage that confer yellow-to-orange colour [60]. The major microalgae species that produce β-carotene are *D. salina*, *D. tertiolecta*, *D. bardawil*, *B. braunii*, *C. nivalis*, *C. acidophila*, *Chlorococcum* sp., *Chlamydocapsa* sp., *Tetraselmis* sp., *C. sorokiniana*, *P. obovate* and *C. striolata* [74].

Microalgae also produce lutein that provides excellent antioxidative properties to prevent or delay the onset of chronic diseases as well as the treatment of cataracts, an eye disease that cause blurry or double vision, faded colours and trouble seeing at night [84]. Lutein is produced by *Chlorella* sp., *B. braunii*, *D. tertiolecta*, *C. nivalis*, *S. almeriensis*, *P. urceolata*, *C. proboscideum*, *C. acidophila*, *N. gelatinosum*, *Chlamydocapsa* sp., *Muriellopsis* sp., *Pyramimonas* sp. and *Tetraselmis* sp. [74]. The market price of lutein is approximately around $910 to $15,000 /kg with an estimated market of around $3.14 million in 2019 [60]. The study by Del Campo, Rodriguez [85] showed that amount of lutein produced by *Chlorella* sp. was less than astaxanthin, causing its market price is higher than astaxanthin.

2.5 Potential of microalgae in enhancement the quality of natural products

Natural products are characterized as the products of natural origins including an entire or part of an organism that has not undergone any processing or treatment other than a simple preservation process such as animals, plants or microorganisms [86]. It is feasible to incorporate high-

value bioactive compounds from microalgae biomass into majority of the products that are consumed on a regular basis to increase its nutritional and functional value.

2.5.1 Pharmaceutical industry

Microalgae have drawn great deal of interest in the recent years despite their potential therapeutic applications. Various research studies shown that the bioactive compounds from microalgae biomass demonstrated medicinal properties such as antimicrobial, antiviral, antifungal, anti-tumour, neuroprotective activities that have been attributed to the pharmaceutical industries [2, 87-89]. Microalgae also synthesize compounds that used for the targeted delivery of anticancer drugs, particularly poorly water-soluble drugs. The diatomaceous earth microparticles extracted from marine microalgae are used for targeted therapies for cancer, for example, colorectal cancer. The compounds were first coated with vitamin B12, a tumour targeting agent, and then filled with anticancer agent, cisplatnum or cisplatin for the efficient delivery of the cancer drug to the targeted site and kill the cancer cells [90]. Furthermore, some of the microalgae strains (e.g., *Chlorella* sp., *Scedesmus* sp., *Chlamydomonas* sp., *Nannochloropsis* sp., *Tetraselmis* sp., *Botryococcus braunii*) were also used for the evaluation for their potential use as photosensitizers in photodynamic therapy or in other word, light-associated treatment of tumours. The study by Jabeen, Reeder [87] showed that these microalgae constituents significantly reduced the proliferation and showed the photo-damage effects on the four cancer cell lines, which were

human lung carcinoma, human breast adenocarcinoma, human prostate cells derived from metastatic site lymph node and human breast adenocarcinoma. Therefore, these microalgae strains were suggested as the potential photosensitizers but the further research, such as *in vivo* and *in vitro* experiments, is needed to confirm their efficacy and efficiency.

The high value compounds produced by the microalgae demonstrated different therapeutic potential that can be integrated in the production of the new drugs with the hope to eradicate the diseases, especially the incurable diseases or infections. Here are some examples of the companies that have derived pharmaceutical products from algae such as Agri Life SOM Phytopharma (India) Limited, Piramal Healthcare, Rincon Pharmaceuticals and Novo Nordisk India Private Ltd. In the future, microalgae extracts can be used to develop vaccine to provide the immunity to the disease or infection such as influenza virus and Zika virus that are the biggest and serious health threats to the people worldwide.

2.5.2 Cosmetics and Personal Care

Microalgae generate compounds that offer a potentially infinite diversity of benefits for the skin including promoting blood circulation, moisturising the skin, activating the cell renewal and the metabolism, increasing the skin's resistance and having an anti-inflammatory effect on the skin. The cosmetics and personal care products including oral care, skin care, sun care, hair care, decorative cosmetics, body care and perfume. Skin care products range from facial cleanser, toner, scrub, essence, moisturizer to mask with different

functions such as hydration, brightening, pore-tightening and anti-aging to restore, protect and regenerate the skin.

Daniel Jouvance is a company that use microalgae to formulate all or part of their product lines including face care, body care and personal care for over 30 years. For an example, Algo [2]IODe by Daniel Jouvance contains a high concentration of marine iodine from *Thalassiosira* to help delay the appearance of new fatty cells and this gives a sliming cure effect for 7 days. *Dunaliella salina* microalgae which rich in marine glycerine had been exploited in AQUACÉANE series and is a powerful moisturizing agent that provides lasting hydration to skin. ÉCLACÉANE by *Noctiluca* microalgae that emit visible and shimmering light on the surface of the ocean, offer the skin a new source of radiance. Furthermore, some of the microalgal derived cosmetics products have been commercialized. For instance, PEPHA®-TIGHT is a pure, unique natural skin firming and highly purified biotechnologically produced extract from *N. oculate* microalgae and was patented to be used in anti-aging creams [91]. Subitec, a German technology company, has utilised the snow algae (*C. signiensis*) as the anti-aging cosmetic ingredient. This high value biotechnological product, the "Snow Algae Powder", was successfully introduced to the market in 2014. The snow algae extracts are effective in reducing the loss in collagen expression in aged fibroblasts, significantly enhanced skin hydration and smoothed crow's feet wrinkles. Besides, ESTÉE LAUDER also launched a microalgal-based product, named Nutritious Microalgae Pore Minimizing Shake Tonic,

formulated with a nutrient-dense microalgae blend of *Chlorella*, *Spirulina* and *Laminaria saccharina* to purify and balance the skin. Sun Chlorella skin cream from Japan is the first skin-nourishing cream that use *Chlorella* to hydrate and nourish the skin. The extracts produced by microalgae also used in producing mask in rejuvenating, moisturising and renewing the skin. For example, B.liv nano bio-cellulose mask is made from Organic *Spirulina*, a blue-green algae from deep sea to hydrate, repair and clarify skin as well as to protect skin from the free radical damage. Besides, pure face mask from L'Oreal Paris is made from combination of clay and red algae to exfoliate, cleanse and refine the skin. The superfood skin reset antioxidant mask from Youth To The People is a rejuvenating cream mask enriched with Seagreens *Spirulina*, bioactive microalgae and other compounds to restore and rejuvenate skin.

Sunscreen is a topical product that protect us against ultraviolet (UV) light and against sunburn. The extracts of *Spirulina* sp. and *Chlorella* sp. are used as ingredients of sun block [42]. *Chlorogloeopsis* sp. extract protects the keratinous tissue by preventing damage from long wave ultraviolet A (UVA) and short wave ultraviolet B (UVB) and thus preventing photo aging, wrinkles formation and skin sagging [92]. *Isochrysis* sp. extracts also applied in sunscreen formulation that contain organic and inorganic filters with SPF 15 to prevent the transmission of UV. Lastly, *Nannochloropsis* sp. was shown effective against transmission of UVA and UVB [92].

Good and beautiful hair gives better impression, so proper hair care is essentially important. *Chlorella vulgaris* and *Spirulina maxima* microalgae

treat the dandruff for stimulation of hair growth [93]. Extracts of *Thalassiosira* sp., *Monodus* sp., *Chlorococcum* sp. and *Chaetoceros* sp. are suggested for the formulations that deal with hair loss [94]. These microalgae extracts regulate the melanogenesis in skin and hair, proliferation of melanocytes, differentiation of keratinocytes and the growth of hair follicles [94]. Oil produced by *Chlorella* sp. demonstrated the ability to make the skin and hair soft and more flexible [95]. A study by Herrmann, Zanella [96] discovered that the methanol extract (BIO1631) of the marine water microalgae *Isochrysis* sp. is highly effective in increasing the percentage of hair follicles in the anagen phase by reducing apoptosis of hair follicle cells in the bulb region. The extract exhibited an average increase in hair shaft elongation, thus is a highly promising candidate for preventing hair loss. However, due to the toxicity of methanol extract, the extracted bioactive product is unreliable for consumption in human body. Other commercial products such as Dove Regenerative Repair Shampoo restores the hair strength by providing nourishment and repair the damaged hair.

The microalgal extract also can be used in decorative cosmetics products such as foundation, concealer, lipstick and eyeliners. For example, Skinicer ocean kiss lipstick is a hygienic lipstick that contains combination of natural hydrating ingredients and microalgae extract, Spiralin. It is clinically proven to have antiviral, antifungal, antibacterial, collagen-forming, cell-regenerative, as well as UV-protective properties. Phycocyanobilin and

phycoerythrobilin produced from *Spirulina* sp. and *Porphyridium* sp. act as pigments for eye-liner and lipsticks [97].

Nowadays, hair colouring or hair dyeing is the popular and common practice for youths' generation. However, the chemicals presence in synthetic hair dye can cause severe damage to hair such as hair fall, hair thinning and easy breakage. Synthetic hair dye may cause allergic reaction in some people and there had news reported that synthetic hair dye may increase the risk of developing cancer. Microalgae extract were shown to produce carotenoids or pigments naturally [24, 78] and thus can be exploited to produce hair colourant or hair dye because the microalgae-based products are regarded as safe as compared to synthetic hair dye.

2.5.3 Food industry

In United States, Food and Drug Administration recognizes the algae-based products or additives as safe [98]. Cookies, biscuits, pasta and dairy products are some of the examples of microalgae-based food ingredients [99]. Dairy products can be incorporated with bioactive compounds from microalgae for health benefits. For example, *Arthrospira* sp. in fermented milk and yogurts can promote the growth and increase the viability of desired probiotics [100]. Apart from incorporation of *Chlorella* sp. into yogurts, *this microalgae* has also been added to cheese to increase hardness and springiness and at the same time, reduce the meltability and cohesiveness [101, 102]. Phycocyanin extracts from *Arthrospira platensis* have been introduced in the production of cookies to increase fibre and protein content [103]. The incorporation of *H.*

pluvialis into the cookies aids to decrease the glycaemic response and at the same time increase the antioxidant capacity [104]. Batista, Niccolai [105] improved the nutritional and potential health benefits by incorporating *C. vulgaris*, *T. suecica*, *A. platensis* and *P. tricornutum* inside the cookies to boost the protein and antioxidants contents.

Pigments and carotenoids from microalgae can be used as food additives as colourings and thickeners [28]. Carotenoids produced from *Dunaliella* sp., *Chlamydomonas* sp., *Chlorella* sp., *Cyanidioschyzon* sp., *Synechocystis* sp. and *S. platensis* act as food colourant [106]. *Arthrospira* sp. was also used as the ingredient for extruded snack food for the purpose of enrichment [107]. The microalgae can be used as a protein ingredient in drinks, for example *Spirulina platensis* in drinks for sport nutrition as *Spirulina platensis* proteins contain all amino acids needed and to increase the athletes' endurance and performance [108].

Pasta is a popular dish in the diet in European and Asian people. Incorporation of microalgae will not affect the cooking and textures of pasta [109]. High microalgae concentration in pasta will improve stickiness and maintain its elasticity [99]. To enhance the nutritional content of fresh spaghetti, *C. vulgaris* and *A. maxima* are added [109]. Integration of *D. vlkianum* and *I. galbana* into pasta provide ω-3 PUFAs and antioxidants with potential health benefits [110-112]. The ability to produce lipids (fatty acids) by microalgae such as I. galbana, P. tricornutum, C. calcitrans, M. subterraneus, S. obliquus, N. gaditana, C. cohnii and T. pseudonana is

another highly interesting aspect in the food industry [113]. The microalgae oil produced have similar composition to the vegetable oils and rich in high-value PUFA such as EPA and DHA. The fatty acids produced such as EPA and DHA can be utilised as nutritional supplement in tablets, capsules, or powders form or for use in nutraceuticals [114]. Microalgae oils are rich in DHA such as *Schizochytrium* sp. have been formulated in the infant products, which is comparable with other traditional sources of DHA.

The study by Ben Atitallah, Hentati [115] demonstrated that addition of *Isochrysis galbana*, *Chlorella minutissima* and *Picochlorum* sp. into canned fish burgers improved the texture, sensory acceptability, nutritional content and functional properties (water and oil holding capacities) as compared to the rest of the formulations. The incorporation also ameliorated the antioxidant activities of the microalgae-supplemented fish burgers. The possible uses of the polysaccharides from microalgae as a prebiotic, source of fibre is another nutritional aspect of interest though these are yet to be developed [116].

There are some examples of the commercial products that utilise microalgae in the production. For instance, the chocolate products, M&M's use *Spirulina* as a natural colouring. Some cooking oil companies use microalgae technology to produce healthy cooking oils for example, Thrive® Algal Oil (*Chlorella*) and Naturel Forte DHA cooking oil by Lam Soon Sdn.Bhd, Malaysia. Lee Biscuits, Malaysia *use Spirulina in the production of crackers*. *Chlorella* and *Spirulina* also used in the production of beverage

(cereal drinks) to increase the nutritional value such as Gold Choice 3 in 1 Chlorella Cereal Drinks, Nature's Own® Instant Brown Rice Cereal with Spirulina, AIK CHEONG Vita Flakes Matcha Spirulina, Super Nutremill 5in1 Spirulina with Oat and Tesco Choice 3 in 1 Spirulina Pre-Mixed Cereal Drink.

However, some properties of microalgae have limited their incorporation in the food products. For example, the antioxidant activity of *Chlorella* sp. and *Arthrospira sp.* can cause the colour and flavour changes in foods and these changes are typically regarded by consumers as undesirable [117]. Next, the microalgae, for instance *Chlorella* sp. and *Spirulina* sp. that confer green colour have restricted their use as colourings in daily-use products because it will adversely influence consumers' perception of the taste and quality. Furthermore, some products have a slight fish flavour when microalgae are added and unfavourable by the consumers [110].

2.5.4 Transportation

The gradual depletion of fossil fuels and the increased emissions of greenhouse gas in the large cities have urged the generation of renewable energy to resolve the problems such as environmental pollution [118]. Therefore, researchers are currently in the progress of developing biofuels from microalgae, a renewable energy that derived from different origins such as animals, plants (e.g., soybean, sunflower, palm oil, coconut). The oil yields and growth rates are vary considerably between different oilseed crops [119, 120]. Microalgae are able to synthesize rapidly and accumulation of large amounts of lipids up to 85% [50, 121]. Microalgae are capable of synthesizing

58,700 litres oil per hectare that can produce 121,104 litres biodiesel per hectare, a transition that apparently promising over conventional fossil fuels and causing global scientific community to pay special attention to the microalgae in the production of biofuels as an alternative sustainable energy [120, 122].

Recent decades have seen the transformation of microalgae biomass into variety forms of biofuel such as biodiesel, bioethanol, biohydrogen, and bio-oil that potentially addresses the current energy crisis [123]. The lipids formed by microalgae contain twice the energy provided by carbohydrates, meaning that the fuel energy content will experience a two-fold increase outstripping other terrestrial plants for the production of biofuel [124]. Microalgae are seen as an ideal feedstock for biofuels due to their advantageous features, such as their phototropic features, such as fast growth rate, high production of biomass and less water and land needed for growth. *Botryococcus braunii* microalgae has the highest acummulated lipid with dry weight of 43%, while the *Tetraselmis suecica* and *Dunaliella tertiolecta* microalgae contains less than 10% [80]. *Chlorella* sp., particularly *Chlorella vulgaris*, is among the microalgae of great interest for biofuels because it can accumulate large amounts of lipids, rapid growth rate and easy cultivation [125]. Importantly, microalgae oil is more economical as compared to the biomass from other oilseed crops and terrestrial plants. Biodiesel derived from microalgae provides similar properties (e.g., density, fire point, viscosity, heat values) compared to petroleum diesel [126]. The biodiesel

derived from microalgae also has high energy density, cleaner as compared to petroleum-based fuels [127]. Due to the environmental impacts caused by other feedstock, till now, there are none of the other potential resources of biodiesel are realistic and practical to replace the sustainability of petroleum diesel alike microalgae.

Through a series of reactions, microalgae oil can be processed into biofuel, bioethanol, biogas, biomethane and biohydrogen [128]. This can be seen through the emerging of the company to do the microalgae-based biofuel research with the hope to solve the energy crisis and replace the fossil fuels at the future, such as Algae. Tec, EcoFuel Laboratories, IBV Biotech, Algaetech International, AlgaeBiotech, Sapphire Energy and etc [129]. For the biofuel productivity, Solix Biofuels able to produce 3000 gallons of biofuel whereas Seambiotic able to produce 100 to 200 gallons of biofuel [130]. Moreover, in 2009, a research program was carried out by ExxonMobil and Synthetic Genomics (SGI) in the development of advanced biofuels from algae. This joint effort is working in the direction of the production of 10000 barrels of algae biofuels by 2025. By adopting the cell engineering technologies at SGI, the research team modified the algae strain to raise the oil content from 20% to more than 40%. From there, the team had discovered a new mechanism to increase the production of oil by discovering a genetic change that could be fine-tuned to regulate the conversion of carbon to oil in the microalgae species, *Nannochloropsis gaditana*. In short, microalgae have the potential to produce renewable fuels such as biodiesel, bioethanol,

biohydrogen, methane and syngas [123]. Microalgae-based biofuels are biodegradable, non-toxic, low emission of sulphur and high release of oxygen [131].

2.5.5 Nutraceuticals/ Health supplement

The main interests of microalgae lipids are the production of the essential fatty acids that exhibit beneficial effects to human health. First thing to be discussed is the use of microalgae oil as nutraceuticals (human supplements) or as food ingredients. Among these, omega-3 and omega-6 fatty acids, for instance, ALA, EPA and DHA are consumed by the humans as the health supplement as it is essential to our health and our body cannot synthesize it. Subsequently, there is an increasing market demand for omega-3 and omega-6 oils. We can obtain these omega oil from the marine fish or the vegetable crops. For example, DHA and EPA present mainly in the fish whereas ALA is mainly found in plant oils, such as soybean, flaxseed and canola oils. However, there is a main issue raised from the consumption of the marine omega-3 oil which is not suitable for vegetarian consumption and unpleasant odour to the consumers. Besides, it will reduce the global fish stock market and the presence of toxins in fish will harm the consumers. The literature studies showed that certain oil-producing microalgae capable of synthesize PUFA at which the microalgae PUFA content is almost same as the marine fish, vegetables, fungi and bacteria. Therefore, microalgae are suggested to become a promising alternative source for these valuable

resources and have been studied broadly as the alternative to EPA and DHA-rich fish oil in the future human consumption.

The market price of the microalgae-based DHA and EPA are approximately USD 50/kg and USD 650/kg, respectively [132], showing that microalgae oil have the business potential to satisfy the customers' needs. *Spirulina* sp. and *Chlorella* sp. are the examples of commercialized oil-producing microalgae. There are a few companies that perform the nutraceuticals research using microalgae oils (omega-3 fatty acids), such as Algae. Tec, Parry nutraceuticals, Fermentalg, Algaetech International, AlgaeBiotech, Algae to Omega Holdings, Alltech Algae and etc [129]. Although the research on the lipids on microalgae in different aspects still ongoing, but there are microalgae oil-based products exist in the market. Pure One™ is one of the supplements that sold in the market as the capsules form that rich in EPA and DHA algal oil. Source Oil Algae Omega 3, extracted from *Schizochytrium* sp., is another approved supplement by Food and Drug Administration. Next, Nordic Naturals Algae Omega, rich in Omega 3 also made from marine microalgae and suitable for vegetarian. The microalgae such as *Cryptecodinium* sp. and *Schizochytrium* sp. are used as poultry feed, particularly chicken to produce omega eggs. In Europe, the purified PUFAs from the microalgae are added as one of the ingredients of infant grade milk [133]. Besides used as supplements, microalgae oil also can be used as cooking oil. One of the examples is Thrive® Algae Oil cooking oil made from algae that contain low level of SFA, as compared to olive oil and avocado oil

and high level of MUFA. In summary, microalgae have the great potential to be exploited as health supplement.

2.6 Harvesting process

One of the key steps in microalgae processing is microalgae harvesting. Microalgae harvesting methods involve the process of isolation or removal of microalgae biomass from the growth medium and can be divided into chemical, physical, electrical or biological methods. The common methods applied for harvesting of microalgae include sedimentation, flotation, coagulation and flocculation, centrifugation, electrophoresis and filtration as summarised in **Table 2.1** [46, 134, 135]. To ensure an effective harvesting efficiency from microalgae, there are a few criteria in deciding which of the harvesting technique is suitable to remove the culture medium from microalgae biomass, such as low production cost, large biomass quantity at mass scale, good quality of harvested compounds, short processing time, species dependent and contaminant-free. In short, despite a wide range of harvesting methods, there is no single technique proved to be effective in harvesting microalgae biomass in terms of cost and recovery rate [134]. The harvesting method can put up the total production cost to 20-30%. Therefore, to achieve the desired liquid-solid separation or biomass recovery rate in a pilot scale, a suitable harvesting method that comprise of one or more of the combination of methods is needed [122].

Table 2.1. Comparison studies between different harvesting techniques.

Harvesting techniques	Advantages	Disadvantages
Filtration (Membrane process)	• High recovery efficiency (70-90%) • No use of power or electricity • Low shear stress	• Time consuming • Occurrence of membrane clogging • High cost and require regular replacement of membrane • Affect by hydrodynamic conditions and membrane size
Chemical coagulation and flocculation	• Fast • Easy to be applied • Less cell damages • Less power or energy use	• High cost • Difficult to separate the coagulant from harvested biomass
Electrocoagulation	• Fast • Easy to be applied • No chemical required	• High costs (equipment and operational cost) • Metal contamination
Bio-flocculation	• Fast • Easy to be applied	• Possibility of mineral or microbial contamination

	• No need energy supply and chemical use	• Difficult to separate the coagulant from harvested biomass
Flotation	• Low cost • Low demand for space • Suitable for large scale • Short operation time	• Needs surfactants • High cost and metal contamination (electroflotation)
Gravity sedimentation	• Low cost	• Long operation time • No suitable for low density cells
Centrifugation	• High recovery rate (achieve up to 95%) • Low risk of contamination • Fast and effective	• High operation and maintenance costs • Expensive if applied in large scale • Risk of cell destruction
Ozonation-dispersed flotation (ODF)	Fast	Expensive

Drying	• Low cost, because it requires sunlight only	• Drying rate depend on climate or weather • Inconsistent sunlight • Risk of compounds loss

2.7 Extraction of bioproducts from microalgae

It is well known that microalgae contain enormous amounts of valuable biomolecules. The extraction of bioactive compounds from microalgae, however, necessitates cellular disruption in order to release the intracellular contents, as the majority of the compounds are found inside the hard rigid cell walls. There are numerous methods for removing the bioactive substances from microalgae biomass. Mechanical extraction, chemical extraction, physical extraction, and enzymatic lysis are the four major categories of extraction methods. Some of the conventional extraction techniques, such as chromatography, are time-consuming and costly because requiring high energy expenditure and costly. Furthermore, some conventional procedures result in the loss of the targeted molecules, lowering the recovery rate. Considering this, scientists are working extremely hard to create a novel separation and purification processes that can be completed in a single extraction step as well as time- and cost-effective. The recyclability, reusability, and toxicity of the extraction solvents used in the extraction process are the important questions to study in this era. Therefore, phase partitioning or also known as “phase distribution” has

been recently emerged and identified as a potential green method for extracting bio-compounds from microalgae [136].

Phase partitioning has proven to be more efficient in recovering biological materials because it demands ambient conditions with high moisture contents in liquid phases. Mass transfer is high due to the low interfacial tension between the two phases. Phase partitioning is simple and easy to operate. It has been employed in a variety of applications, including the recovery of biopharmaceuticals, enzyme and protein purification, and extractive bioconversion. The disadvantage is the difficulty to forecast the phase equilibrium and the degree of the product partitioning because there is limited understanding of the mechanics underlying the partitioning process. The partitioning effects of phase partitioning systems are affected by various factors such as the molecular weight and concentration of the solvents, ionic strength of salt phase, pH, temperature, and the number of cycles [137].

2.7.1 Liquid biphasic flotation (LBF)

Recently, many studies have focused on a well-known bioseparation method known as liquid biphasic system (LBS) or aqueous two-phase system (ATPS), to separate and purify biomolecules from the organisms, especially microalgae. LBS is the contemporary direction of research in separation and purification field because LBS possesses the advantages such as rapid, affordable, simple to scale up, and environmental-friendly. A biphasic layer will be created when the mixing of two incompatible liquids occurs above the

critical conditions and separates by an interfacial layer. Depending on the selectivity and general features of the phase-forming components, a physico-chemical interaction can easily adapt the desired biocompounds to be partitioned to either the top or bottom phase. Additional supported technologies, including bubbling and ultrasonic have been incorporated into the LBS to increase the effectiveness of biomolecule separation [138-140].

The bubble-assisted LBS or also known as liquid biphasic flotation (LBF) is a combination of LBS and solvent sublation that uses air bubbles (such as nitrogen and oxygen) to aerate the medium made up of an organic solvent and an aqueous salt solution in order to facilitate the separate the biomolecules. The hypothesis is based on the phenomenon of surface-active biomolecules having a sorption mechanism between the surfaces of the floating air bubbles [139]. The LBF system is advantageous since it has a high concentration coefficient and extraction yield, simple operation, taking up less time and space, environmental friendliness and can work continuously.

2.7.2 Three Phase Partitioning (TPP)

In the LBF system, each top and bottom phase of the system can only extract one component. Accordingly, a new approach is suggested named three phase partitioning (TPP). TPP is similar to the conventional salting out because they use the same salt, ammonium sulphate [141]. TPP involves the use of t-butanol and ammonium sulphate to precipitate the desired biomolecules from the aqueous solution. The discovery that many enzymes

continue to function in mixtures of t-butanol and water in 1972 is one of the foundational studies for TPP development [142]. T-butanol is widely used for the separation of proteins from microbial, plant, or animal sources. T-butanol also stabilizes protein structure and inhibits the enzyme activities as well as the protein interactions [141]. T-butanol is typically used as the organic phase because it has a high boiling point and low flammability, which makes it ideal for phase recycling [143]. TPP was first applied for the litre scale precipitation of crude cellulases and other enzymes [144]. TPP is frequently helpful for isolation on a semi-micro, millilitre volume scale at downstream processing.

The TPP process, which involves adding salt to an aqueous solution containing the desired product before adding t-butanol to create three phases. The three phases are the bottom salt phase, middle interface protein precipitate, and top organic phase. Depending on the amount of ammonium sulphate added, protein that was initially in the aqueous phase may separate into a third phase which is halfway between the lower aqueous and upper t-butanol phases. This is the fundamental idea behind the three-phase partitioning technique. The desired biomolecules can partition to any phase in TPP because of the operational conditions and its physicochemical properties [145]. **Table 2.2** demonstrates the examples of biomolecules extracted using TPP which is previously reported in the literature. The mass transfer phenomenon determines the partitioning behaviour of the targeted product, and increasing mass transfer can improve the purity and partitioning of the compounds [146]. To enhance the mass transfer of desired products,

assisted technologies, such as ultrasonication probes and ionic liquid, has been effectively used [147-149].

The benefits of TPP are simple and easy to operate with short processing time. TPP is a cost-effective technique since ammonium sulphate and t-butanol are low-cost chemicals and can be reused for the next experiment. Contrary to chromatography, which dilutes the purified protein, TPP concentrates and purifies the protein. Compared to simple salting out techniques, the purification fold achieved in TPP is significantly higher. TPP can be performed at ambient temperature (room temperature), whereas organic solvent precipitations require low temperatures. Hence, the process conditions in TPP are more favourable for the preservation of organic compounds. Next, the mild TPP conditions prevent the denaturation of proteins. Lastly, TPP can be scaled up or down to semi-microlevels or litre scale. TPP is applicable to direct crude cultures that contain cell debris and no any pre-treatment procedures, such as centrifugation, are necessary [150].

Table 2.2 Examples of biomolecules from various organism extracted using TPP.

Organisms	Biomolecules	Experimental conditions	Reference
Papaya peels	Proteases	- Ratio of extract to t-butanol = 1.0:0.5	[151]

		- 55% $(NH_4)_2SO_4$ - Recovery rate = 89.4% - Purification = 10.1-fold	
Leaves of *Ipomoea palmata*	Peroxidase	- Ratio of extract to t-butanol = 1:1 - 30% $(NH_4)_2SO_4$ - Activity recovery = 160% - Purification = twofold	[152]
Jatropha curcas L. seed	Oil	- Ratio of t-butanol and 30% $(NH_4)_2SO_4$ = 1:1 - Combination of sonication and enzyme treatment (Protizyme™) at pH 9 - Oil yield = 97% oil yield	[153]
Mango kernel, soybean, and rice bran		- Pretreatment with protease enzyme, Protizyme™ - Oil yield = 98% (soybean), 86% (rice bran) and 79% (mango kernel)	[154]

Bacillus sphaericus MTCC 3672	Fibrinolytic enzyme	- Pre-treatment =Ultrasound irradiation (25 kHz, 150 W power with 40% duty cycle for 5 min) - 80% $(NH_4)_2SO_4$, extract to t-butanol ratio =0.5 - Recovery = 65% - Purity = 16.15-fold	[155]
Microalgae	Lipid (*Chlorella* sp.)	- TPP system: dipotassium hydrogen phosphate and ethanol, 12.5g/L microalgae - Extraction yield = 69%	[156]
	Lipid (*Nannochloropsis* sp.)	- CO_2-responsive deep eutectic solvents and 20% $(NH_4)_2SO_4$ were used for TPP - CO_2 and N_2 were bubbled after extraction. - Formation of three phases: DES-rich top phase, middle	[157]

	solid phase, salt-rich bottom phase - Extraction yield = 172.3 mg/g - CO_2-responsive DES can be recycled and reused, with 10.6% reduce in the yield	
Lipid (*Chlorella saccharophila*)	- Optimized conditions: 30 % $(NH_4)_2SO_4$, 1:0.75 ratio of extract and t-butanol - Pre-treatments: Sonication probe with recovery of 69.05%; High-pressure homogenization with recovery of 89.91% - t-butanol layer: Approximately 1.26% carotenoids - Middle layer: Around 12 % of protein	[158]
Lipid (*Chlorella vulgaris*)	- Cell disruption by water-plasma	[159]

	- Lipid recovery =74.34%	
Docosapentaenoic acid (*Schizocytrium limacinium* SR21)	- Cell disruption: microwave (650 W for 25 s) - Optimized conditions: 37.5% n-hexane, 13.33% potassium acetate – Yield: DHA (98.9%), total fatty acids (98.4%), β-carotene (97.0%), astaxanthin (94.9 %), protein (94.8%), flavonoids (93.3%).	[160]
Protein *(Chlorella vulgaris*)	- Cell disruption: Microwave (120s, duty cycle 80%, 100 W) - Optimized condition: 30% $(NH_4)_2SO_4$, 1:1 ratio of extract to t-butanol, 0.5% biomass concentration - Separation efficiency = 67.2% - Recovery yield = 63.2%	[161]
Protein (*Chlorella pyrenoidosa*)	- Enzymatic treatment (Stargen™ and Carezyme™)	[162]

	- Optimized parameters: extract to butanol ratio (1:1.5), t-butanol and 40 % $(NH_4)_2SO_4$, incubation time (20 min)	
Protein (*Chlorella vulgaris* FSP-E)	- Cell disruption: Ultrasound (100% power, 10 min, 35 kHz, duty cycle of 80%) - Optimized conditions: 50% $(NH_4)_2SO_4$, extract to t-butanol (1:2), biomass (0.75 wt%) - Separation efficiency: 74.59% - Yield: 56.6%	[145]

2.8 Obstacles faced during extraction of biomolecules

During the lipid extraction from microalgae biomass, the liposoluble intracellular pigments such as chlorophyll will be co-extracted into crude oil and thus affecting oil quality and limit its large-scale application. Chlorophyll is insoluble in water but readily dissolved in organic solvents including ethanol, acetone, and chloroform [17] and these solvents are used commonly for extraction of biomolecules, especially lipid. To date, there are less studies regarding the removal of chlorophyll from microalgae and the developed

methods were inefficient. **Table 2.3** provided examples of numerous techniques used to remove pigments, particularly chlorophyll from the microalgae.

Table 2.3 Methods for removing chlorophyll from microalgae.

Microalgae species	Chlorophyll removal method	Findings	Reference
Scenedesmus sp.	Treatment with bleaching earth at 80 °C and under vacuum	Chlorophyll and total carotene concentrations reduced from 4296.7 ppm and 1918.9 ppm to 40.3 ppm and 199.0 ppm, respectively, with removal efficiencies of 99.1% for chlorophyll and 89.6% for total carotene. The colour of the refined biodiesel was red or orange.	[18]
Scenedesmus sp.	Saponification method using sodium hydroxide, NaOH with	96% of the chlorophyll in the biomass was removed. The chlorophyll-reduced biomass could be used to produce high-quality orange clear oil.	[19]

	methanol, ethanol, and acetone	Particularly, the amount of neutral lipids and fatty acid saturation levels rose. The pigments' composition shifted towards carotenoids.	
Mixed cultures (Majority: *Chlorella* and *Scenedesmus* sp.)	Chlorophyll precipitation with the addition of 0.5 M sulphuric acid to form a solid precipitate	In contrast to direct transesterification, the wet lipid extraction method achieved chlorophyll removal through previous precipitation, removing or significantly lowering chlorophyll contamination of the crude biodiesel.	[163]
Chlorella vulgaris strain UTEX 2714	Chemical chlorophyll removal using hypochlorite solution	The culture was bleached white using 1% hypochlorite concentration. The remaining cultures with a bleach concentration of greater than 0.06% also became colourless after 20 minutes of incubation. It's possible that	[164]

		more hypochlorite will be needed to completely bleach the cultures with higher chlorophyll concentration. Only the chlorophyll appeared to be degraded after hypochlorite bleaching, which may be because the cell wall was still visible and so prevented cell lysis.	
Chlorella vulgaris	Solvent extraction methods using methanol, acetone, dimethylsulfoxide (DMSO)	DMSO was discovered to be a reliable solvent for chlorophyll extraction. Using DMSO has the advantage of not requiring incubation of biomass with DMSO for complete removal of chlorophyll, as other solvents such as acetone and methanol do.	[165]
Chlorella homosphaera	Saponification method (98% ethanol and 1%	67.5% of the chlorophyll was removed from the microalgae. The lipid extraction yield from	[166]

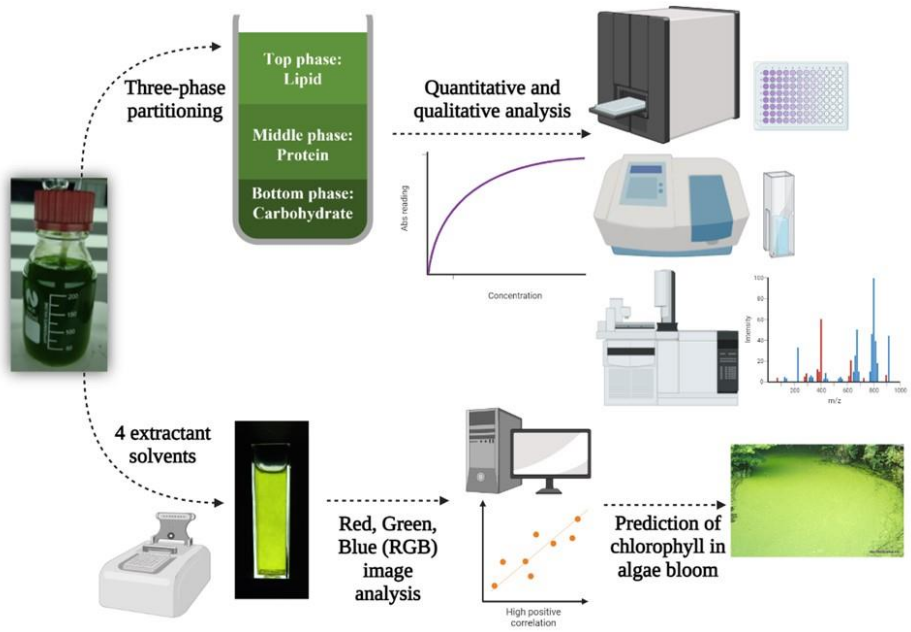
	NaOH at 70 °C for 1 hour)	chlorophyll-treated biomass was 30.35%, corresponding to a 44.2% loss of lipids from 54.39% in the control samples without chlorophyll removal. The observed decrease in recoverable lipids could be attributed to a loss of 26.54% of biomass during the chlorophyll removal process.	
Chlorella vulgaris	Hydrodynamic cavitation- aided Fenton reaction	The initial chlorophyll content was 80.1 mg/L. The chlorophyll removal efficiency by the hydrodynamic cavitation treatment was 16.6%. When paired with Fenton-like reaction (1.0% hydrogen peroxide and pH 3), the chlorophyll removal rate was significantly increased, reaching 97.2%. The Fenton-hydrodynamic cavitation reaction bleached the lipid-	[167]

		solvent complex's colour from dark green to transparent.	
Chlamydomonas sp. JSC4	Removal during the transesterification reaction involving hexane-to-methanol with a ratio of 6:1 at 45 °C for 15 min together with solid base catalyst, Sr_2SiO_4	When Sr_2SiO_4 concentration was greater than 0.12 g per 12 mL of oil-containing hexane solution, the chlorophyll was almost completely removed, corresponding to the catalyst loading of 0.40 g- Sr_2SiO_4/g-microalgae oil. The colour of Sr_2SiO_4 changed to green during the transesterification process, which could be due to the adsorption of chlorophyll from the microalgal oil extracts on the catalyst surface. As a result, reusing of the Sr_2SiO_4 is extremely challenging.	[168]
Monoraphidium sp. GK12	Acid treatment (sulphuric acid, hydrochloric acid,	When the microalgal extract was treated in 0.01-0.1 N H_2SO_4 or HCl, a considerable	[169]

	phosphoric acid, and acetic acid)	amount of chlorophyll was eliminated, but astaxanthin was lost from the extract in 0.02-0.1 N H_2SO_4. Thus, treatment in 0.01 N H_2SO_4 or HCl was found to be effective for removing 80% of chlorophyll without the loss of astaxanthin. H_3PO_4 and CH_3COOH, on the other hand, were unable of removing chlorophyll effectively.	
Mychonastes homospaera	Saponification method (NaOH: ethanol in various ratio)	NaOH: ethanol with ratio of 7:3 at 60 °C and 90 min of reaction time had the highest chlorophyll removal percentage, which was 93.25%. The total lipid loss was the least with maximum saturated fatty acids and mono-unsaturated fatty acids yield.	[170]

Chlorella sorokiniana (UTEX 1602)	Phosphoric acid discoloration pre-treatment: Combination of crude microalgal oil with 1% (v/v) pure phosphoric acid at 100 °C for 30 minutes.	Total chlorophyll and carotenoid levels in crude oil may be reduced from 2290 and 2254 ppm to 7 ppm and 754 ppm, respectively, showing a removal effectiveness of 99.7% and 66.6% for chlorophyll and carotenoid pigments. The refined oil was orange in colour, indicating the presence of carotenoids.	[171]

CHAPTER 3 TRIPHASIC PARTITIONING OF MIXED *SCENEDESMUS* AND *DESMODESMUS* FOR NUTRIENTS EXTRACTION AND CHLOROPHYLL COMPOSITION PREDICTION FOR ALGAE BLOOM



This chapter evaluates the biochemical composition of mixed microalgae species, *Desmodesmus* sp. and *Scenedesmus* sp. using TPP system. This chapter also covers an innovative and inexpensive method based on red-green-blue (RGB) image analysis to estimate the microalgae chlorophyll content. The digital images were acquired using smartphone camera. The colour index was then evaluated using software and associated with chlorophyll concentration significantly. This chapter consists of thesis-version of work published in the Environmental Technology (https://doi.org/10.1080/09593330.2022.2150094).

Triphasic partitioning of mixed *Scenedesmus* and *Desmodesmus* for nutrients extraction and chlorophyll composition prediction for algae bloom

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3.1 Abstract

Overgrowth of microalgae will result in harmful algae blooms that can affect aquatic ecosystem and human health. Therefore, quantitation of chlorophyll pigments can be used as the indicator of algae bloom. However, it is difficult to monitor the geographical and temporal distribution of chlorophyll in the aquatic environment. Accordingly, an innovative and inexpensive method based on red-green-blue (RGB) image analysis was utilized in this study to estimate the microalgae chlorophyll content. The digital images were acquired using smartphone camera. The colour index was then evaluated using software and associated with chlorophyll concentration significantly. Regression model using RGB colour components as independent variables to estimate chlorophyll concentration was developed and validated. The Green in RGB index was the most promising way to estimate chlorophyll concentration in microalgae. The result showed that acetone was the best extractant solvent with high R-squared value among the four extractant solvents. Next, isolation of useful biomolecules such as proteins, fatty acids, polysaccharides and antioxidants from the microalgae has been recognized as an alternative to regulate algae bloom. Microalgae are shown to produce bioactive compounds with a variety of biological activities that can be applied in various industries. This study evaluates the biochemical composition of mixed microalgae species, *Desmodesmus* sp. and *Scenedesmus* sp. using liquid triphasic partitioning (TPP) system. The findings from analytical assays revealed that the biomass consisted of varied concentration of carbohydrates,

protein, and lipids. Phenolic compounds and antioxidant activity were high, at 60.22 mg/L of phenolic compounds and 90.69% scavenging activity respectively.

Keywords: Red Green Blue (RGB) colour model; Chlorophyll concentration; Antioxidant; Fatty acids; Polysaccharides; Microalgae

3.2 Introduction

Due to pollution and eutrophication over the past few years, there has been an increase in concern regarding the accumulation of the algae population in the marine ecosystem which can result in algae bloom. Algae bloom may lead to environmental issues such as increases the turbidity and water pH that can decrease the potability of water and affect aquaculture sector. In addition, the naturally occurring phycotoxins by algae bloom can cause detrimental effects on human health through the consumption of affected seafood, inhalation and direct skin contact [172]. The decomposition of the algae bloom may also negatively impact the ecosystem through the release of toxins and excessive greenhouse gases [173, 174]. Chlorophyll concentration is a metric to determine the quantity of algae proliferating in the water and used to categorise the trophic condition. Thus, high concentration of chlorophyll, chlorophyll a and chlorophyll b, is correlated with the early sign of algae bloom. The conventional method to determine the chlorophyll content of the microalgae is through solvent extraction, followed by spectrophotometric instrumentation. This method is accurate and precise, but time-consuming,

expensive, and labour-intensive. This method also requires destructive sampling and dilution of sample if the absorbance value obtained is too high (exceed 1.0) as well as many calculation steps to obtain the chlorophyll concentration. The disadvantage using spectrophotometric analysis is the presence and interference of the other molecular samples that can affect the spectral features of the sample. Even while the other interference molecules can absorb wavelength at the rates comparable to those of chlorophyll pigments, the qualification of the chlorophyll will be a challenge.

Hence, the research on rapid and non-destructive chlorophyll determination method have been proposed. The majority of prior research has applied remotely sensed images from satellites and aircrafts or assimilation of these data into the algorithms to develop one software, to determine chlorophyll concentration [175]. For instance, Johansen, Beck [176] assessed the effectiveness of 29 algorithms to estimate the concentration of chlorophyll-a using satellite-based spectral imager data that can be utilised as an indicator of the possibility of algae bloom. Mozo, Morón-López [177] built a data-driven chlorophyll-a soft sensor using machine learning approach and automatic high-frequency monitoring technology to monitor algae bloom. Laneve, Bruno [178] compiled all the data on the condition of Lake Pertusillo including Sentinel-2A satellite data to monitor algae bloom. However, the satellite remote-sensing images have spatial resolution of ten to over a thousand meters. As a result, it is challenging for the development of algorithms using satellite data to determine and predict

the chlorophyll concentration in an accurate and precision way as well as requires expertise knowledge [175].

For that reason, this has driven the further development of image-processing techniques, for example using Red-Green-Blue (RGB) colour model, to estimate the chlorophyll concentration from the plants [179-181]. Utilizing three colour sensors per pixel to measure light intensity in the red (R), green (G), or blue (B) spectrums, the RGB colour model enables the registration of the colour of any pixel of a picture of an object [179, 180]. Riccardi, Mele [180] estimated chlorophyll content in quinoa and amaranth leaves using RGB components analysis of digital photos captured with single-lens reflex camera. At the same time, do Amaral, Vieira Silva [179] evaluated the chlorophyll content and physiological condition of trees using RGB component analysis from the digitalized images via scanner. To the best of our knowledge, the use of RGB colour model incorporated with the digital images from smartphone camera has ever been investigated to quantify the microalgae biomass concentration but is limited for chlorophyll estimation in microalgae [182]. Hence, the possibility to use a quick and non-destructive method for chlorophyll determination is crucial. The purpose of this study is to assess the applicability of RGB components based on a single, readily available image taken with smartphone camera. To get the chlorophyll reference values, the chlorophyll is extracted using four types of organic solvents from the microalgae solution, followed by spectrophotometric analysis. Regression models using RGB colour components and chlorophyll

concentration as the variables have been developed and evaluated to determine the reliability of the model. The algorithm based on image processing is created regardless of the changes in the camera settings (resolution, noise, scale, perspective). The captured images are extracted into the RGB colour components, and each colour index is estimated. Colour index is then computed through the formula given where R, G and B stand for the values for red, green, and blue colour, respectively. The image processing and analysis tool is conducted using Microsoft Visual Studio. This study serves as a platform for the development of a rapid and reliable digital analysis system in determining and estimating the microalgae chlorophyll contents using image analysis.

To control algae bloom in the aquatic ecosystem, the isolation of bioactive compounds from the microalgae can be an alternative solution. Microalgae are the photosynthetic autotrophs that can synthesize various high value-added bioactive compounds, such as polyunsaturated fatty acids, amino acids, enzymes, lutein, fucoxanthin, glycerol, polysaccharides, that have high bioactivities such as antibacterial, antioxidant, antifungal, anticancer and other beneficial properties. Thus, microalgae have attracted the attention of researchers worldwide by further expanding their potential in various industrial areas [183]. The conventional separation techniques used for the extraction of biomolecules are membrane separation, column chromatography, precipitation and crystallization. These drawbacks of these separation methods are the use of toxic organic solvents, time-consuming

(multi-step procedure) and are expensive. Therefore, a green, time- and cost-efficient technique named three phase partitioning (TPP) is employed to overcome the mentioned drawbacks. Chew, Chia [161] and Chia, Chew [184] employed TPP to extract and purify protein from *Chlorella vulgaris*. TPP is evolved from aqueous two-phase system and liquid biphasic system that involved the use of two solvents, salt (ammonium sulphate) and alcohol (t-butanol). The end results are the formation of three phases instead of biphasic formation at which t-butanol and lipids will be at the top layer, protein precipitates at the middle layer and an aqueous bottom carbohydrates layer. The advantages of TPP are efficient, scalable, short processing time and can operate in a one-step procedure [161, 184, 185]. Due to the operating conditions and physicochemical characteristics of the specific biomolecules, it can partition into any of the phases [185, 186].

This study has two major objectives, which are to propose a RGB model in evaluating the chlorophyll content using smartphone camera, and to assess the bioactive compounds present in the mixed microalgae species. The mixed microalgae species, *Desmodesmus* sp. and *Scenedesmus* sp. is used in this present study. The morphology of marine microalgae, *Desmodesmus* sp., are visibly round in shaped with rigid shells, lack of flagella and non-motile. The epistructures morphology are warts, spines or single elongated tubes, and rosettes on the cell surface [35]. On the other hand, the freshwater algae, *Scenedesmus* sp. are found in flat or curved shaped. The cells of genus *Scenedesmus* are elongate, cylindrical, irregular,

dactylococcoid or ellipsoid [187]. To date, there is a lack of literature studies on the assessment of the biochemical properties of these microalgae species.

3.3 Materials and Methods

3.3.1 Materials

Freeze dried microalgae powder, *Desmodesmus* sp. and *Scenedesmus* sp. was obtained from the Swedish University of Agricultural Sciences (a generous gift from Dr. Francesco Gentili). The microalgae were cultivated as previously reported [188] in Umeå, northern Sweden (63° 86' N) in an open pond placed close connection with the local combined heat and power plant (Umeå Energi, Umeå Sweden) and fed with untreated municipal wastewater (Vakin Umeå). All the chemicals used in this experiment were of analytical grade.

3.3.2 Methodology

Chlorophyll quantification

The chlorophyll content was extracted by using solvents as followed: 90% acetone, ethanol, dimethyl sulfoxide and methanol. 30 mg of wet microalgae biomass was added with the solvents, followed by incubation in the water bath at 50 °C for 30 minutes. The liquid was centrifuged three times at 9433 g force for 10 minutes. If the absorbance reading exceeded the linear range of instrument detection, dilution is needed by adding the respective solvents until the absorbance reading was ranged from 0.2 to 0.8. The

chlorophyll content of the samples was evaluated using UV-spectrophotometer. The respective extractant solvent was used as the blank. The quantity of chlorophyll (chlorophyll a and chlorophyll b) were determined using the artificial intelligence model by inserting the equations (**Equation 3.1 to Equation 3.4**) as illustrated below [189] into the model:

$$\text{Total chlorophyll content for 90\% acetone} = Chlorophyll_{\ a}\ (12.25A_{663} - 279\ A_{646}) + Chlorophyll_{\ b}(21.5A_{646} - 5.1A_{663}) \text{.....Equation 3.1}$$

$$\text{Total chlorophyll content for ethanol} = Chlorophyll_{\ a}\ (13.36A_{664} - 5.19\ A_{649}) + Chlorophyll_{\ b}(27.43A_{649} - 8.12A_{664}) \text{.....Equation 3.2}$$

$$\text{Total chlorophyll content for dimethyl sulfoxide} = Chlorophyll_{\ a}\ (12.47A_{665} - 3.62\ A_{663}) + Chlorophyll_{\ b}(25.06A_{663} - 6.5A_{665}) \text{ .....Equation 3.3}$$

$$\text{Total chlorophyll content for methanol} = Chlorophyll_{\ a}\ (16.72A_{665} - 9.16\ A_{652}) + Chlorophyll_{\ b}(34.09A_{652} - 6.5A_{665}) \text{.... Equation 3.4}$$

RGB data acquisition

In this study, Redmi Note 9s mobile phone camera was used to capture the image. The 6.67-inch screen of this smartphone has a resolution of 2400 x 1080 pixels. The main camera used to capture the images is a 48MP ultra-high resolution primary camera, with specifications of f/1.8 aperture as well as 1.6µm 4-in-1 Super Pixel and 0.8µm pixel size. The position of the camera, lighting and background information can influence the data collection; hence, the conditions of individual image were kept standardized for better accuracy of colour index and determination of chlorophyll content. The image set-up was illustrated in **Figure 3.1** using a black box. After the absorbance reading,

the cuvette filled with chlorophyll solution was placed inside the black box. The camera was placed 1 cm from the hole carved at the black box to capture the visual image of the microalgae culture. The images of chlorophyll were captured using the camera and performed in multiple shots to collect data. There was total 800 pictures with individual absorbance readings being taken to ensure the accuracy and reliability of data.

Image processing

The obtained images were pre-processing through two steps which were grayscaling by converting the images to monochrome images followed by thresholding that involved the conversion of images to binary images to increase the success rate of segmentation step. Next, the processed images were undergoing segmentation, pixel selection and conversion between colour models, for instance RGB to CMYK (Cyan, Magenta, Yellow, and Key) and HSL (hue, saturation, and lightness) to obtain values of colour index. Lastly, regression analysis was used for data analysis to compute R-square value which indicated the correlation between colour index and biomass.

Training of the model

The data obtained was the absorbance reading of different extractant solvents and there was a total of 800 data used for data training. The concentration of the chlorophyll was calculated using the equations by employing various machine learning algorithms, 80% of the obtained data (640 individual data) was used for training of the models. Light Gradient Boosted Machine (LightGBM) was selected to train and select AI model with

satisfactory performance. The remaining 20% of the data (160 individual data) was used for data validation or for testing.

Regression modelling

The colour index obtained from the digital image of microalgae chlorophyll were regressed against the corresponding chlorophyll concentration.

Experimental setup - TPP

TPP was applied to separate and extract biomolecules from the microalgae. The procedure was adapted from previous literature studies with minor modification [161, 185]. The system was generated by dissolving 1% w/w of microalgae biomass in distilled water, followed by mixing the system with 30% w/v $(NH_4)_2SO_4$ in a beaker. Equivalent volume of t-butanol was then added to the mixture and stirred using magnetic stirrer at 350 rpm for 1 h. The mixture was left to stand for 30 min for the formation of three phases after stirring process. Each phase (top, middle and bottom) was pipetted into respective centrifuge tubes for further reaction tests to quantify the biochemical composition. Tris-HCl buffer (pH 7.40) was added to the middle phase (consisting of protein) before analysis test. The separation efficiency of all three phases as stated in **Equation 3.5** was calculated.

Separation efficiency (E, %) of protein or carbohydrates:

$$E = \frac{V_I C_I}{V_U C_U + V_I C_I + V_B C_B} \text{ ... ... Equation 3.5}$$

V_U: Upper phase volume (mL)

V_I: Intermediate phase volume (mL)

V_B: Bottom phase volume (mL)

C_U: Upper phase concentration

C_I: Intermediate phase concentration

C_B: Bottom phase concentration

Analytical methods

The bottom phase (carbohydrate) was tested using phenol-sulphuric acid method, by mixing 2 mL of sample from bottom phase with 1 mL of 0.5 % phenol and 2 mL of concentrated sulphuric acid in the glass vial. The absorbance of the samples was read at 490 nm using ultraviolet–visible (UV) spectrophotometer (UV-1800, Shimadzu, Japan). For the middle phase, Thermo Scientific™ Pierce™ Bicinchoninic Acid Protein Assay Kit was used to estimate the protein concentration with bovine serum albumin as the standard. 25 µL of sample or standard solutions were pipetted into a microplate well, followed by addition of 200 µL of the working reagent to each well and 30 min incubation at 37 °C. The absorbance was read at 562 nm using a microplate reader. The top phase was determined quantitatively and qualitatively using gravimetric method and gas chromatography (Perkin Elmer Clarus 690 GC, USA) with flame ionisation detector (GC-FID). The samples were injected into an Agilent Technologies analytical laboratory instrument column (InertCap Pure-WAX, GL Sciences) (30 m ×0.25 mm × id 0.25 µm). The standard used in

this study was Fatty Acid Methyl Ester Mix, C4-C24 certified reference material (Sigma-Aldrich) [190].

The antioxidant activity in the crude microalgae extract was evaluated using total phenolic content (TPC) assay and Trolox equivalent antioxidant capacity assay (TEAC) [191]. The experiments were conducted in dark condition and room temperature. The standard involved in TPC assay was gallic acid (GAE) solution in different concentration by mixing 500 µL diluted Folin-Ciocalteu (F-C) with 100 µL sample and 2 mL of $NaHCO_3$ solution, followed by absorbance reading at 725 nm. For TEAC, 3.8 mL of diluted 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) solution was pipetted into 100 µL of sample, followed by 6-min incubation and absorbance reading at 734 nm. The 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity was determined via **Equation 3.6**:

$$\text{Percentage of scavenging } (\%) = \frac{\text{Control} - \text{sample/standard}}{\text{Control}} \times 100 \quad \text{.....Equation 3.6}$$

3.3.3 Statistical analysis

The experiments were triplicated, and the average values were reported. At least three replicates were performed. All the data was expressed as mean ± standard error of mean. The significance test was established by conducting analysis of variance or t test using SPSS Statistics.

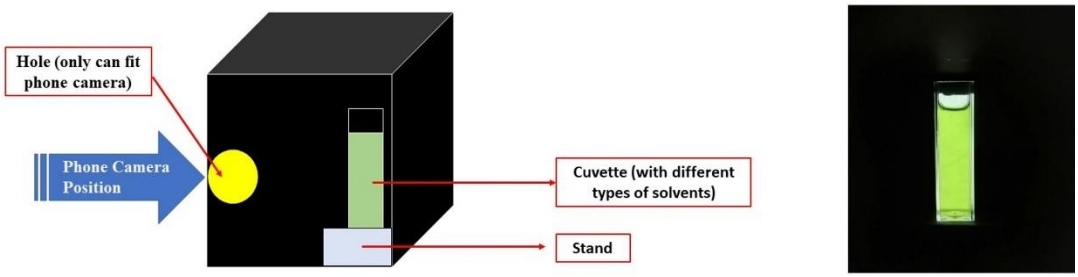

Figure 3.1 Black Box Setting and the examples of image taken using camera phone. The cuvette filled with solution consisted of chlorophyll and extractant solvent is placed in black box and the position of camera is fixed and constant throughout the experiment.

3.4 Results and Discussion

RGB imaging-based estimation of chlorophyll content

Microalgae are rich in chlorophyll which can divided into two main types, chlorophyll a and chlorophyll b. According to the findings, amount of chlorophyll a was higher than chlorophyll b in the mixed microalgae species because the primary pigment for light harvesting is chlorophyll a that transforms light energy into chemical energy and chlorophyll b is acting as accessory pigment. Next, the laboratory analytical of the chlorophyll extraction from the microalgae via organic solvents produced a wide range of pigment values as shown in **Table 3.1**. Acetone had the highest value of chlorophyll content as compared to ethanol, methanol and DMSO. A different result was observed in previous literature [192, 193]. Amin, Chetpattananondh [192] study showed that methanol had excellent capability

to extract chlorophyll from microalgae *Chlorella* sp. as compared to ethanol and acetone. Next, the study by Parveen, Duddela [193] showed that ethanol was more effective in extracting chlorophyll from *C. reinhardtii* and *C. vulgaris*. The extraction of chlorophyll by different solvents depended largely on the chemical nature of biomolecules (chlorophyll-a, chlorophyll-b and carotenoids) in the particular microalgae species [189], while for the mixed species in present study, acetone was found to be best and suitable extractant solvent.

The present finding demonstrated a correlation between RGB index of the image and the microalgae chlorophyll content. As compared to R and B, the G value was discovered to have the strongest correlation with microalgae chlorophyll because chlorophyll a absorbed light of violet, blue and red and mainly reflected it in green colour. That was the reason chlorophyll concentration was measured spectrophotometrically in the range of 650–675 nm. A similar conclusion was reached by study from Özreçberoğlu and Kahramanoğlu [194] that G had the highest correlation with white LED or daylight illumination. After obtaining the colour index of samples per extractant solvents, regression analysis was performed against the total concentration of chlorophyll a and chlorophyll b for these four solvents. The regression model was developed with one dependent variable (concentration) and one independent variable (colour index). The best regression model was linear. R-square value indicates the correlation between colour index and microalgae biomass (chlorophyll concentration).

Our results demonstrated that methanol showed the strongest linear relationship with colour index whereas ethanol showed the weakest linear relationship. The RGB regression model showed the greatest R-square value of 0.475, 0.318 and 0.173 for acetone, DMSO and methanol, respectively with significant F-statistics ($p<0.05$). Nonetheless, R-squared value does not convey the reliability of a model and cannot be used to determine whether the regression model fits adequately to the data set [195]. Therefore, residual plots can be used as a further tool to evaluate the validation of regression model. If the points in a residual plot are randomly distributed along horizontal axis, it can be said that the linear regression model is appropriate for the data [196]. Through the regression analysis, the residual plot (**Figure 3.2**) by the four extractant solvents showed a random arrangement. This proved and confirmed that the linear regression model in this present study was appropriate.

The present investigation revealed that the proposed approach enabled the simultaneous collection of data from microalgae to estimate the chlorophyll amount instead of the conventional solvent extraction and ultraviolet–visible spectroscopy method. The estimation of chlorophyll concentration is crucial to monitor the growth of algae bloom. Each colour component was obtained from the acquired digital image, allowing the quantification of chlorophyll content in rapid and non-destructive way. The advantages of using digital camera or smartphone camera to capture the image in RGB analysis are its cost-effectiveness and availability. The

limitation in the chlorophyll determination is the uniformity of the ambient lighting conditions at the time when data is collected. Besides, there is currently no consensus on the best digital camera used for sampling. Depending on the application, smartphone camera has the advantages, in terms of flexibility, accessibility and cost, over other type of advanced digital cameras and scanner, such as GoPro camera, digital single-lens reflex (DSLR) camera, point-and-shoot camera, and charge-coupled device (CCD) camera. However, as compared to smartphone camera, these advanced cameras equipped with the sensors can produce high resolution images, waterproof but are expensive. Despite this, the technology innovation has increased the resolution of smartphone cameras with enhancement of complementary metal oxide semiconductor (CMOS) image sensors in the mobile phone. Furthermore, it is essential to take note in this study that this is device-dependent, since the range of colour index may vary according to the phone specifications. The broadening of data collection for the adaptation of different camera specifications can be performed to allow the general usage of the automated system and to overcome the device-dependent problem. In short, the applicability of these findings provides a useful substitute to monitor chlorophyll in the occurrence of algae bloom for the simplicity of field data collection.

Extraction of biomolecules using TPP

To control algae bloom, one of the ways is to isolate microalgae from the algae bloom for the extraction of biomolecules, such as fatty acids, amino

acids, polysaccharides, antioxidants and enzymes. Microalgae biomass has been shown to produce a variety of biomolecules with various beneficial properties that can be applied in various industries as alternative substitute, for example food and beverage, pharmaceutical, nutraceuticals and biofuel. Consider the example of polysaccharides, especially ulvan (a type of sulphated polysaccharides), have shown to exhibit medicinal properties, which includes anticancer, antifungal, antihyperlipidemic, hepatoprotective, antiprotozoal, and anti-inflammatory [183]. In addition, microalgae is able to produce proteins for biopharmaceuticals products or therapeutic protein, for example, recombinant proteins, edible vaccines, antibodies, immunotoxins, antimicrobial agents and others [197]. In the same way, there are numerous literature studies on antioxidative potential of microalgae that is similar with medicinal plants. The use of synthetic antioxidants, for instance, butylated hydroxytoluene, butylated hydroxyanisole, and propyl gallate, can have possible toxic and carcinogenic effects. Therefore, a low-cost, safe, and natural antioxidant will be more ideal.

There have been numerous downstream processing methods being introduced to extract biomolecules from the microalgae, which are precipitation, solvent extraction, filtration, electroporation, supercritical fluid extraction and chromatography techniques. However, these conventional techniques require large amounts of organic solvents, time-consuming, expensive and some approach requires expertise knowledge. Furthermore, some pH or temperature-sensitive biologically active substances can lose

their activity during the extraction at extreme conditions. Thus, an alternative separation named aqueous biphasic system (ABS) is developed to separate desired compounds through the formation of two immiscible aqueous phase comprises of protein and carbohydrate. ABS is a simple, environmental-friendly, rapid, able to operate continuously, time- and cost-effective system, making them applicable to numerous applications [191, 198-202]. Due to mentioned benefits, ABS is then advanced to TPP in the extraction of biomolecules, that are lipid, carbohydrate and protein, simultaneously in a single step [161, 184, 185]. TPP employs the addition of t-butanol and ammonium sulphate to the raw material or crude extract to extract and purify targeted molecules at room temperature which results in the formation of three phases, comprises of lipid, carbohydrate and protein. TPP approach is also suitable to be applied directly with crude suspension and is an environmental-friendly approach as the solvents used for extraction can be recovered and recycled for subsequent cycles. Ammonium sulphate and t-butanol are considered as “safe” solvents as compared to organic solvents such as hexane and chloroform [203]. The advantages, such as ease of operation, efficient, continuous operation, and mild operating conditions, make TPP suited for the separation and purification of bioactive compounds from the microalgae. However, the solvents used in TPP are not suitable to isolate certain compounds for example blood protein as reported by Gagaoua and Hafid [204] that high concentration of t-butanol can change protein structure. IgG antibodies and proteins with concentration less than 5 µg

cannot be isolated using TPP. Thus, it is essential to check the properties of targeted compounds and their suitability with t-butanol and ammonium sulphate before extraction using TPP.

In this experiment, the mixed microalgae species were subjected to three phase partitioning to form three layers, top, middle and bottom phases, that comprised of lipid, protein and carbohydrates, respectively. TPP was selected because the targeted biomolecules would not lose their activity during the reaction with ammonium sulphate and t-butanol. Thus, the accuracy of the results would not be affected. The biochemical characterization was conducted, and results were presented in **Table 3.2**. The carbohydrates content in the mixed microalgae species was found to be 1.17 ± 0.04 mg/mL. The standard curve of the carbohydrate was obtained with R^2 = 0.9946 and the equation: $y = 0.395x + 0.1201$ where y is the absorbance of starch at 490 nm and x is the concentration of starch. The carbohydrates content of these microalgae species was high as compared to the protein and lipid portion. The study by Visca, Di Caprio [205] showed that the carbohydrates content of *Scenedesmus* sp. (30.5%) was high as compared to the *Chlorella* sp. (17.7%). Sriram and Seenivasan [206] also reported that the maximum carbohydrate content of 25.4% was obtained under constant light intensity and 16:8 h light: dark cycle for *Desmodesmus* sp. A similar conclusion was also reached by the study by Ansari, Gupta [207] that *Scenedesmus obliquus* cultivated in wastewater produced carbohydrate and lipid yield of 19.80% and 25.39%. Besides, protein content found in the

study was 0.453 ± 0.003 g/L with the separation efficiency of 95.43%. The standard curve of the protein was obtained with $R^2 = 0.9962$ and the equation: $y = 0.0011x + 0.0446$ where y is the absorbance of bovine serum albumin at 490 nm and x is the concentration of bovine serum albumin. However, the presence of non-protein elements may influence the absorbance reading through bicinchoninic acid assay. Bicinchoninic acid protein assay was used to determine protein concentration due to its stable colour complex, high sensitivity and applicability over a broad range of protein concentration. However, cysteine, tyrosine and tryptophan residues may affect the bicinchoninic acid assay. These amino acids, copper chelating agents and reducing agents will affect the accuracy of the results. In future developments, electrophoresis and chromatography methods can be thoroughly investigated to determine the protein content in microalgae.

The gravimetric analysis showed that the lipid content of microalgae was about 6.57% of the microalgae biomass. According to **Figure 3.3,** the lipid profile obtained from gas chromatography showed that the highest peak was seen for C11 (0.1870 mg/g), followed by C16 (0.0459 mg/g), C12 (0.0053 mg/g) and lastly C20 (0.0012 mg/g). The gas chromatography analysis results revealed that the fatty acid methyl esters profiles of the mixed microalgae species contain an abundance of saturated fatty acids (undecanoic acid, lauric acid) as compared to unsaturated fatty acids (palmitoleic acid, eicosapentaenoic acid). The highest weight was found in C11 (undecanoic acid) whereas the lowest was C20 (eicosapentaenoic acid). The performance

of this assay can be enhanced by increasing the sensitivity in the detection of compound or mass spectrometry methods to verify the compounds. This was supported by the findings from Chaudhary, Khattar [208] that showed *Desmodesmus subspicatus* biomass contained high saturated fatty acids (47%) under nitrogen limitation as compared to monounsaturated fatty acids (30%) and polyunsaturated fatty acids (23%). Gour, Chawla [209] studied *Scenedesmus dimorphus* biomass and stated that it had higher occurrence of saturated fatty acids (53.04%) than monounsaturated fatty acids (23.81%) and polyunsaturated fatty acids (19.69%). This result tied well with previous studies wherein lipid and carbohydrate yield of *Scenedesmus obliquus* was around 16.0% and 20.4 %, respectively with the biomass concentration of 71.6 mg L^{-1} [210, 211]. In pharmaceutical industries, different fatty acids possess their individual functions, such as omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid have important roles in cardiovascular disease risk management. However, the mixed microalgae species used in this study comprised of lower amounts of eicosapentaenoic acid as compared to saturated fatty acids, undecanoic acid. Undecanoic acid or undecylenic acid can be used as antifungal agents in topical antifungal formulas for the treatment of dermatomycosis, onychomycosis, and tinea pedis and is normally extracted from *Candida albicans* [212]. Therefore, the abundance of this fatty acid in these mixed microalgae species suggests it as an alternative microorganism to extract undecanoic acid for making active ingredients for topical treatments.

Together, the present findings from TPC and TEAC showed that antioxidant activity of mixed microalgae species was satisfactory [191]. A linear calibration curve of gallic acid with R^2 value of 0.9907 was obtained by measuring using the gallic acid equivalents equation of $y = 0.0027x + 0.1404$, whereby y is the absorbance at 765nm, and x is the concentration of total phenolic compounds in mg per mL of the extract. The results showed that the microalgae species exhibited about 60.22 mg/L of phenolic compounds (15.65 mg gallic acid equivalents/g). The phenolic content in this extract was considered high and is expected to produce satisfactory antioxidant activities. Trolox equivalent antioxidant capacity assay measures the ability of antioxidants to transform 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical, a blue-green chromophore at which its intensity will decrease if antioxidants are present. From the Trolox equivalent antioxidant capacity assay, the percentage of scavenging found was 90.69% (5.6625 µmol Trolox equivalents/g) with the standard curve of $y = 0.0993\ x + 1.6436$ ($R^2 = 0.9982$). A similar effect has been previously observed in other studies [213, 214]. For example, for *Scenedesmus* sp., the antioxidant capacity was found to be 3.71 ± 0.11 µmol Trolox eq. g^{-1} dry weight in ethanol/water mixture by 2,2-diphenyl-1-picrylhydrazyl whereas the value was 5.40 ± 0.28 mg gallic acid eq. g^{-1} dry weight for total phenolic content [213]. Besides, the study by Safafar, Van Wagenen [214] showed that the phenolic contents in *Desmodesmus* sp. was 7.72 ± 0.08 mg/g and 24.26 ± 0.60 Trolox equivalent/g in biomass concentration of 1.0 mg/mL. These two past literature

studies indicated that the two microalgae species exhibited high antioxidant activities. Phenolic compounds were found as major contributors to the antioxidant activity in all antioxidant tests [214]. Hence, high total phenolic content was correlated with high antioxidant capacity. Previous studies showed that the antioxidant capacity was strongly related with the total flavonoid content and total phenolic compounds of the extract [213, 215]. Different extracts using different types of solvent can also be used to evaluate and compare the antioxidant activity in this microalgae species. Overall, the application of TPP as the platform for bioseparation procedures is versatile and functional.

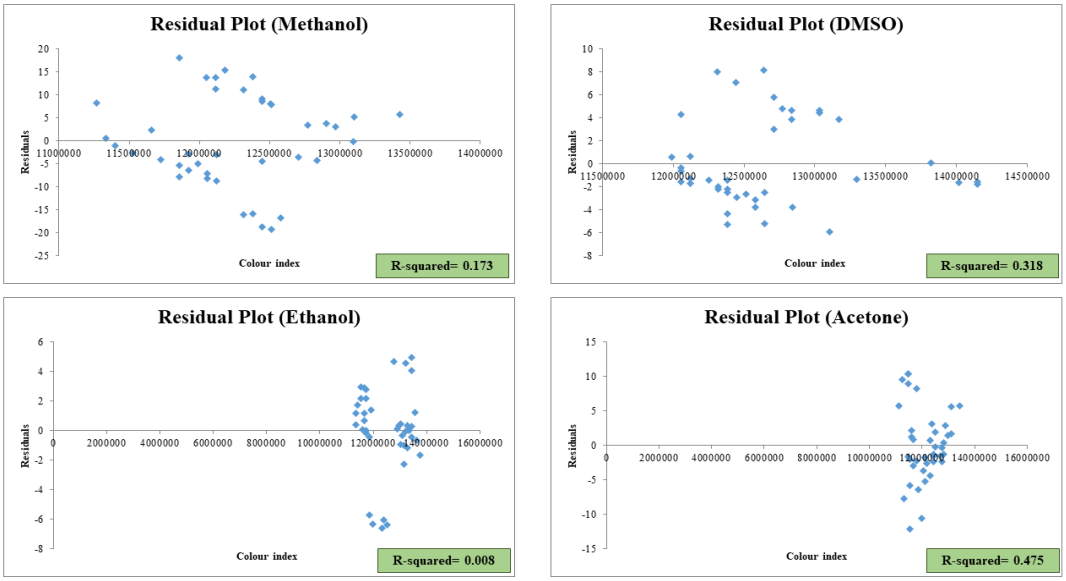

Figure 3.2. Residual plot and R-squared value of chlorophyll extraction using different solvents (Methanol, DMSO, Ethanol, Acetone).

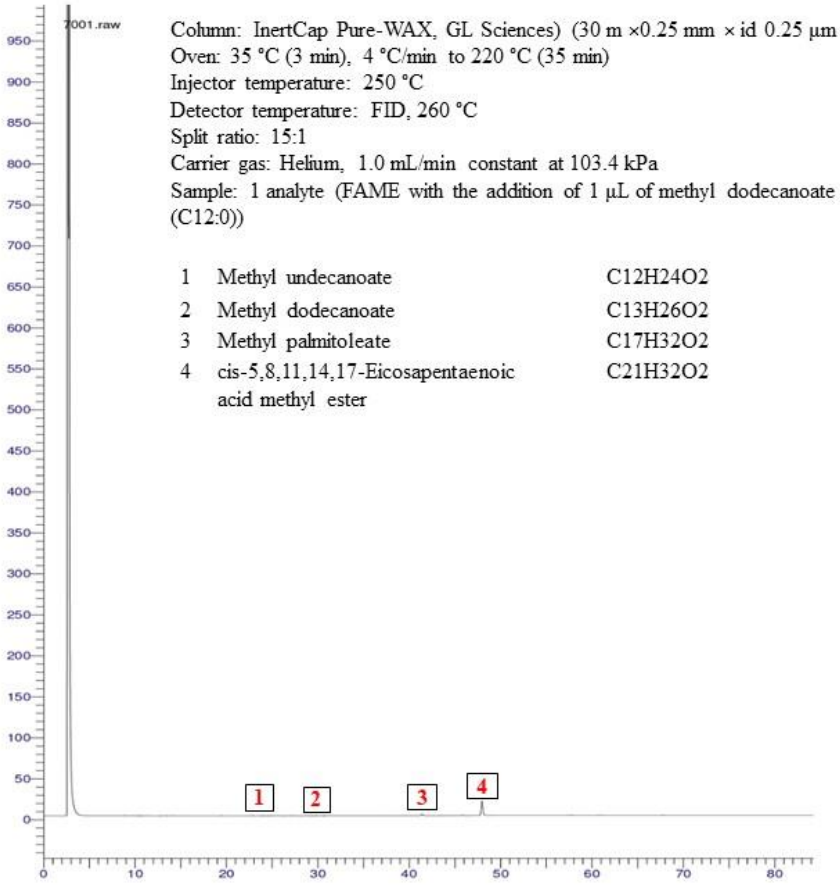

Figure 3.3. GC-FID Lipid Analysis of Mixed Microalgae Species.

Table 3.1 Laboratory analytical determination of chlorophyll content in microalgae using various organic solvents.

Organic solvents	Concentration of chlorophyll (µg/ml)	
	Min	Max
Methanol	9.832	29.632

DMSO	10.609	26.045
Ethanol	6.727	19.820
Acetone	15.320	28.350

Table 3.2 Results findings of biochemical characterization of three phases (top, middle and bottom) obtained using TPP.

Phases	Biomolecules	Test	Results
Top	Lipid	Gravimetric analysis	0.032 ± 0.0043 mg/g
Intermediate	Protein	BCA assay	0.453 ± 0.003 mg/mL
Bottom	Carbohydrates	Phenol sulphuric acid	1.17 ± 0.04 mg/mL

3.5 Conclusion

The present investigation revealed that the rapid automated non-destructive technique using RGB model in determining the chlorophyll contents. The colour index extracted from the image together with absorbance reading for each solvent were used to develop the detecting model in forecasting the

chlorophyll concentration. The results were summarized as acetone worked well to extract chlorophyll, while ethanol displayed the least linear association with the colour index, methanol displayed the strongest. The findings showed that a good fit regression was achieved between the chlorophyll concentration and RGB colour index. The use of digital image segmentation in RGB colour model to determine the chlorophyll content is still a relatively new technology and its primary challenges are the absence of standardised protocols for image acquisition and choice of the most appropriate index. In the future, larger sample size and more machine learning models could be employed to train accurate data. This will enable a more reliable relationship between the concentration of chlorophyll and colour index as well as the development of a model for the purpose of recognizing the microalgae chlorophyll concentration. In addition, the accuracy and precision of the RGB method could be improved with advanced and high-quality sampling method, for instance GoPro, DSLR and CCD cameras. Overall, this research study holds the promise in a practical and affordable approach to estimate chlorophyll concentration in microalgae species in monitoring the occurrence of algae bloom. The potential application of TPP in the separation and extraction of lipids, protein and polysaccharide from the microalgae simultaneously in a single step is proved effective.

CRediT authorship contribution statement

Doris Ying Ying Tang: Conceptualization, Methodology, Investigation, Writing - original draft.; Sia Yuk-Heng: Investigation, Formal analysis.; Huong-Yong

Ting: Formal analysis.; Kit Wayne Chew: Writing - review & editing, Methodology.; Shir Reen Chia: Methodology, Writing - review & editing.; Francesco G. Gentili: Resources, Writing - review & editing, Supervision.; Pau Loke Show: Funding acquisition, Supervision.; , Zengling Ma, Mukesh Kumar Awasthi: Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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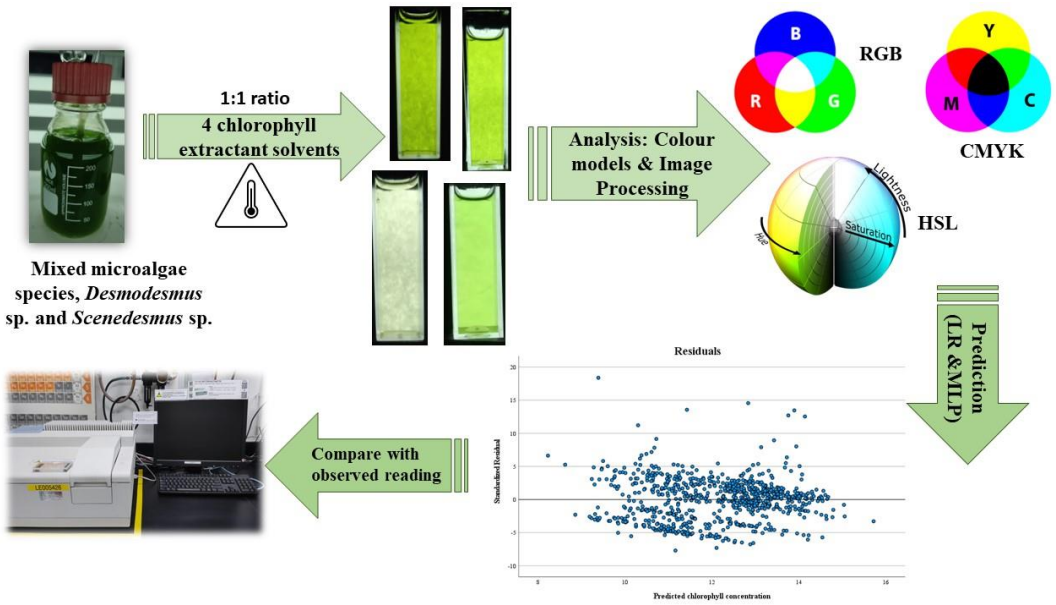
CHAPTER 4 APPLICATION OF REGRESSION AND ARTIFICIAL NEURAL NETWORK ANALYSIS OF RED-GREEN-BLUE IMAGE COMPONENTS IN PREDICTION OF CHLOROPHYLL CONTENT IN MICROALGAE



This chapter introduces a rapid and non-invasive method based on colorimetry of digital images to predict microalgae chlorophyll concentration by using smartphone camera for image acquisition. The chlorophyll concentration can be estimated by analysing the spectral information of each colour component. This chapter consists of thesis-version of work published in the Bioresource Technology (https://doi.org/10.1016/j.biortech.2022.128503).

Application of Regression and Artificial Neural Network Analysis of Red-Green-Blue Image Components in Prediction of Chlorophyll Content in Microalgae

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4.1 Abstract

This study presented a novel methodology to predict microalgae chlorophyll content from colour models using linear regression and artificial neural network. The analysis was performed using SPSS software. Type of extractant solvents and image indexes were used as the input data for the artificial neural network calculation. The findings revealed that the regression model was highly significant, with high R^2 of 0.58 and RSME of 3.16, making it a useful tool for predicting the chlorophyll concentration. Simultaneously, artificial neural network model with R^2 of 0.66 and low RMSE of 2.36 proved to be more accurate than regression model. The model which fitted to the experimental data indicated that acetone was a suitable extraction solvent. In comparison to the cyan-magenta-yellow-black

model in image analysis, the red-green-blue model offered a better correlation. In short, the estimation of chlorophyll concentration using prediction models are rapid, more efficient, and less expensive.

Keywords: Chlorophyll; Microalgae; Prediction; Red-green-blue colour model; Multilayer perceptron; Regression

4.2 Introduction

Microalgae are sustainable and fast-growing photosynthetic organisms, which can produce high value bioproducts and biofuels that have broad application possibilities in renewable energy, biopharmaceutical, and nutraceutical industries [183, 216]. Besides, microalgae are also frequently used in wastewater treatment to detoxify both organic and inorganic pollutants in wastewater streams [217]. Chlorophyll is one of the valuable biocompounds that are present in microalgae. Chlorophyll a and b differ primarily in how they participate in photosynthesis. Chlorophyll a is the main pigment involved in the process, whilst chlorophyll b is an auxiliary pigment that gathers energy to transfer to chlorophyll a. In the development of a variety of chlorophyll-based supplemental products, measurements of the chlorophyll pigments concentration are crucial. Additionally, chlorophyll level can be used to determine the growth and physiological status of microalgae during cultivation process [215, 218-222]. Managing the microalgae culture without visual inspection could result in a failure to identify the unhealthy microalgae cells [223]. Besides, chlorophyll level can

also indicate the extent of algae bloom in the aquatic ecosystem as it is one of the most serious ecological issues affecting the organisms in marine ecosystems.

Spectrophotometric analysis is the conventional technique for measuring the amount of chlorophyll in the microalgae extract by measuring the reflectance at a specific wavelength and utilising formulas to determine the concentration of the chlorophyll. This quantification method has been proven to be frequently erroneous due to the overlapping of absorption spectra of other pigments, for instance carotenoids, with chlorophyll. The standard curve can be ineffective if the chlorophyll integrity is compromised by the changes in temperature, pH and other unknown circumstances [223]. Besides, research carried by the researchers offers numerous formulated simultaneous equations [224-228], making it unclear to which equation should be used to calculate the chlorophyll concentration. The large-scale field applications of traditional chlorophyll analysis are deemed as time-consuming and limited in technological advancement.

Various rapid and non-destructive alternative approaches to estimate chlorophyll content, for instance imaging techniques using the colour models and machine learning approaches, have been explored over time. These imaging techniques are increasingly attracting the attention from the researchers worldwide due to their high throughput and real time applicability. When it comes to visual based quantification, colour models like RGB (Red, Green, and Blue), CMYK (Cyan, Magenta, Yellow, and

Black), and HSL (hue, saturation and luminance/ brightness) play a crucial role [229]. Red-green-blue is utilised in digital displays whereas cyan-magenta-yellow-black is used for printing. As for the hue-lightness-saturation colour model, hue is the pure colour tone in the colour spectrum, saturation describes how the colour tone is combined with grey, and luminance describes the brightness level of the resulting colour [230].

Linear regression and artificial neural network are frequently employed to build the prediction models. In the past years, researchers were looking for the optimum regression correlation and artificial neural network design to predict the condition or detect the concentration of desired biomolecules [231]. In this study, both models are used to predict microalgae chlorophyll content from the image index using appropriate colour model. Linear regression is a prediction tool that can assist in forecasting chlorophyll concentration based on a variety of factors. The model is built by fitting a linear equation to observe the data pattern. The ability to show a relationship between variables is the main advantage of this statistical method, even though no causal mechanism is indicated. The setup of regression model is simple and easy to implement [232]. Next, artificial neural network or "universal approximator" which can capture linear and non-linear relationships between statistical inputs and output data models has been applied as a computational tool for predictive modelling of chlorophyll level in the organisms [233, 234]. Artificial neural network is unquestionably a potent and robust tool for identifying non-linearity, despite

that it has a drawback as a black-box model in which there is little explanatory insight into the role of the independent parameters in the prediction [235]. The weighted connections between nodes are adjusted as the part of artificial neural network learning process which resembles how brain neuronal networks work. The model is trained using experiment-generated data. Furthermore, by providing the input and output data, it is possible to calculate an error based on the target output and the actual output [232, 236, 237]. Even if the input differs slightly from that used in the experiments, it can predict the outcome in real time [238]. With R value of more than 0.98 and very low RMSE, it can be concluded that the model's predictive capacity is accurate with excellent agreement. The artificial neural network model can be used to optimize the procedure while employing input values that are different from those used in the experiments within the same range. By experimentally confirming the outputs that were expected for these inputs, a different set of parameter optimal levels can be discovered [237].

The use of artificial neural network and linear regression approaches to forecast and ascertain the chlorophyll concentration has not been addressed in any of the prior research studies. Therefore, this study compares the effectiveness of linear regression and artificial neural network in predicting the chlorophyll concentration in microalgae species using images of colour. The images are captured using smartphone camera. A multilayer perceptron which is one of the fields of artificial neural network is

chosen in this study. Multilayer perceptron is chosen because it is widely used and has a number of layers made up of neurons and their connections which has the capacity to compute the weighted sum of its inputs, which is followed by the application of an activation function to produce a signal that will be sent to the following neuron. Multilayer perceptron uses types of extractant solvents (acetone, ethanol, methanol and dimethyl sulfoxide) and image index as the input data for calculation. The datasets are divided into three sections: training, testing and validation. This study will be very helpful for the identification of chlorophyll concentration in the industry for bioproducts manufacturing, and monitoring of microalgae growth in industrial scale as well as detection of algae bloom.

4.3 Materials and Methods

4.3.1 Materials

Dr. Francesco Gentili from Swedish University of Agricultural Sciences kindly donated the freeze-dried microalgae powder (*Desmodesmus* sp. and *Scenedesmus* sp.) for the use in this study. The chemical reagents (ethanol, acetone, methanol, DMSO) and consumables (beaker, test tubes, pipette tips, cuvette) were all of analytical grade.

4.3.2 Determination of chlorophyll concentration

The whole procedure was illustrated in **Figure 4.1**. The measurement of chlorophyll concentration was conducted through absorbance measurement using a UV-vis spectrophotometer (Shimadzu UV-1800). The experiment

was conducted in dark condition. The microalgae chlorophyll was extracted using different extraction solvents. The microalgae biomass was mixed with 5 mL of extraction solvent, followed by incubation in water bath at 50 °C for 30 minutes. Lastly, the mixture with total volume of 10 mL was centrifuged at the relative centrifugal force of 5030 x g for 10 minutes to allow the easy separation of microalgae biomass from the supernatant that consisted of chlorophyll. The absorbance was measured at multiple wavelengths between 400 and 700 nm against the blank in order to ascertain the chlorophyll concentration. The chlorophyll concentration (µg/mL) was measured using equations as shown below [239]:

- $Chlorophyll\ a = (11.64\ A_{663} - 2.16\ A_{645} + 0.10\ A_{630})\ v/IV$ ……. Eq (4.1)
- $Chlorophyll\ b = (20.97\ A_{645} - 3.94\ A_{663} - 3.66\ A_{630})\ v/IV$ ……. Eq (4.2)

Where A = the absorbance at wavelength after removing the sample absorbance at 750 nm against a blank of the solvent used; v = volume of extractant solvent used (mL); I = spectrophotometric cell length (cuvette) (cm); V = sample volume (mL)

4.3.3 Image acquisition

The present study captured images using a smartphone camera (16 MP, f/2.2, 27mm) in 8-bit red-green-blue mode. A Blackbox was designed for the imaging purposes at which the distance between the phone camera lens and the Blackbox was approximately 5 cm. The imaging Blackbox was

illuminated by the surrounding light in the laboratory (LED tube lights, 18 W, colour temperature 6500 K). During the study, the cuvette comprised of chlorophyll were loaded into the Blackbox. Total of 1000 images were taken for every cuvette with chlorophyll with the resolution of 2250 x 4000 pixels. Images were saved in .jpeg format. First, the acquired images were converted to monochrome image, followed by conversion to binary image with black and white colour only to enhance the segmentation step. Next, the images were segmented and cropped to separate the cuvette from the background. When selecting the colour pixel, only the middle 25% to 75% of the width and height was selected. The images were analysed using three colour models, which were red-green-blue, cyan-magenta-yellow-black and hue-lightness-saturation colour models. Different colour features were extracted from the images at which the colour will provide the necessary information to estimate the chlorophyll concentration in microalgae. The analysis was done using Microsoft Office Excel 12.0 Data Analysis Tool Pack.

4.3.3.1 Red-green-blue colour model

Red, green, and blue light, which were used in red-green-blue imaging, have the wavelength ranges of 615–620 nm, 530–540 nm, and 460–470 nm, respectively. The colour components were made up of pixels with red-green-blue values that range from 0 to 255 for each band.

4.3.3.2 Cyan-magenta-yellow-black colour model

The R, G, B values were divided by 255 to change the range from 0...255 to 0...1 to obtain cyan-magenta-yellow-black values. In this study, the values will be converted into percentages.

- K = 1-max (R', G', B') ……. Eq (4.3)
- C = (1-R'-K) / (1-K) ……. Eq (4.4)
- M = (1-G'-K) / (1-K) ……. Eq (4.5)
- Y = (1-B'-K) / (1-K) ……. Eq (4.6)

4.3.3.3 Hue-lightness-saturation colour model

From red-green-blue data, spectral characteristics including H, S and L were computed.

- **Saturation, S**

$$S = \begin{Bmatrix} 0 \\ \Delta \quad ,\Delta = 0 \\ 1 - |2\,L - 1|\ ,\ \ \Delta <> 0 \end{Bmatrix} \text{……Eq (4.7)}$$

- **Hue, H**

$$H = \begin{Bmatrix} 0°\ ,\Delta = 0 \\ 60°\ \times \left(\frac{G\prime - B\prime}{\Delta} mod 6\right)\ ,C_{max} = R' \\ 60°\ \times \left(\frac{B\prime - R\prime}{\Delta} + 2\right)\ ,C_{max} = \ G' \\ 60°\ \times \left(\frac{R\prime - G\prime}{\Delta} + 4\right),\ C_{max} = B' \end{Bmatrix} \text{…… Eq (4.8)}$$

- **Lightness**, **L**

$$L = (C_{max} + C_{min}) / 2 \text{ ……. Eq (4.9)}$$

4.3.4 Selection of suitable colour index for mathematical modelling

The colour index of three models for different extraction solvents were calculated and recorded. Data analysis was conducted through the Pearson correlation coefficient to study whether the colour index was correlated as well as the strength of the association with the extraction solvents. R-squared value that indicated the correlation between colour index and chlorophyll concentration was determined to select the best model for subsequent analysis.

- $Index_{RGB}=(R*256*256)+(G*256)+B$ ……. Eq (4.10)
- $Index_{CMYK}=(Y*101*101*101)+(K*101*101)+(C*101)+M$…… Eq (4.11)
- $Index_{HSL}=(H*101*101)+(S*101)+L$ …….. Eq (4.12)

4.3.5 Statistical modelling

This study used linear regression and multilayer perceptron to estimate the chlorophyll concentrations from the colour index. The statistical modelling was conducted using IBM® SPSS® Statistics 28.

4.3.5.1 Linear regression

Linear regression was used to investigate the linear relationship between the response and predictor variables, which were colour index and

chlorophyll concentration. In general, the model can be presented as follows:

$$y_i = a + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_n X_n \text{ …….. Eq (4.13)}$$

Where y_i = Expected or estimated value for chlorophyll; a = intercept (the value of y_i when all X's = 0); B_1, B_2, B_n = Coefficients of the predictor variables; X_1, X_2, X_n = Predictor variables

4.3.5.2 Artificial neural network modelling and construction

Multilayer perceptron training algorithm was chosen for non-invasive chlorophyll concentration prediction because it was effective at simulating functional relationships. Multilayer perceptron was a feedforward system that used a set of inputs and outputs to produce a set of results. First stage was data collection and systematization. For the learning process, artificial neural networks required a large and varied source of training data. Hence, the database comprised of 1000 samples. The learning dataset must be chosen carefully because a poor choice could skew the entire artificial neural network training. The input to the model consists of two parameters which were the colour index and type of extractant solvents. The first input was calculated based on the equations in Section 2.4 whereas last input measured directly. The datasets were divided into two groups. 70% of data was utilised as training data, whereas the remaining 30% were used for measuring prediction accuracy of the model. Three layers of the multilayer perceptron, including input, one hidden and the output layers, were chosen. Multilayer perceptron model used in this study had few neurons in the input

layer (red, green, blue and red-green-blue index with and without extraction solvent) and one neuron in the output layer (total chlorophyll concentration). The system has at least one hidden layer. The maximum number of hidden layers was set to a reasonable three. The dependent variable, in this case, chlorophyll concentration was selected. Colour index and the solvent types were selected as covariate or factor and rescale to standardised. Training type was in batch mode and scaled conjugate gradient was used as optimization algorithm. Training options were initial lambda, initial sigma, interval centre and interval offset. The predicted chlorophyll concentration was calculated.

4.3.5.3 Goodness of fit and predictive performance

To identify the model with the greatest performance, the measured and predicted microalgae chlorophyll concentration were compared in terms of Pearson's correlation coefficient (R) and coefficient of determination (R^2). The degree of linear relationship between predicted and experimental data was indicated by the correlation coefficient. R value was between -1 and 1. If R = 0, there was no linear relationship; but, if R = 1 or -1, there was, respectively, an ideal positive or negative linear relationship. The error's variance was measured using the coefficient of determination, which has a range of 0 to 1. Less error variation was indicated by higher R^2 values [240]. Residual of the predicted chart and observed chart was also calculated. Predictive performance of the models was evaluated using root mean squared error (RMSE). The residuals of the best fitted linear regression and

multilayer perceptron were then examined to determine their normal distribution, homoscedasticity, and outlier existence in order to assess model assumptions.

$$\text{RSME} = \sqrt{\frac{1}{n}\sum_{1=1}^{n}((X_{observed} - X_{\ predicted})^2} \ldots\ldots \text{Eq (4.14)}$$

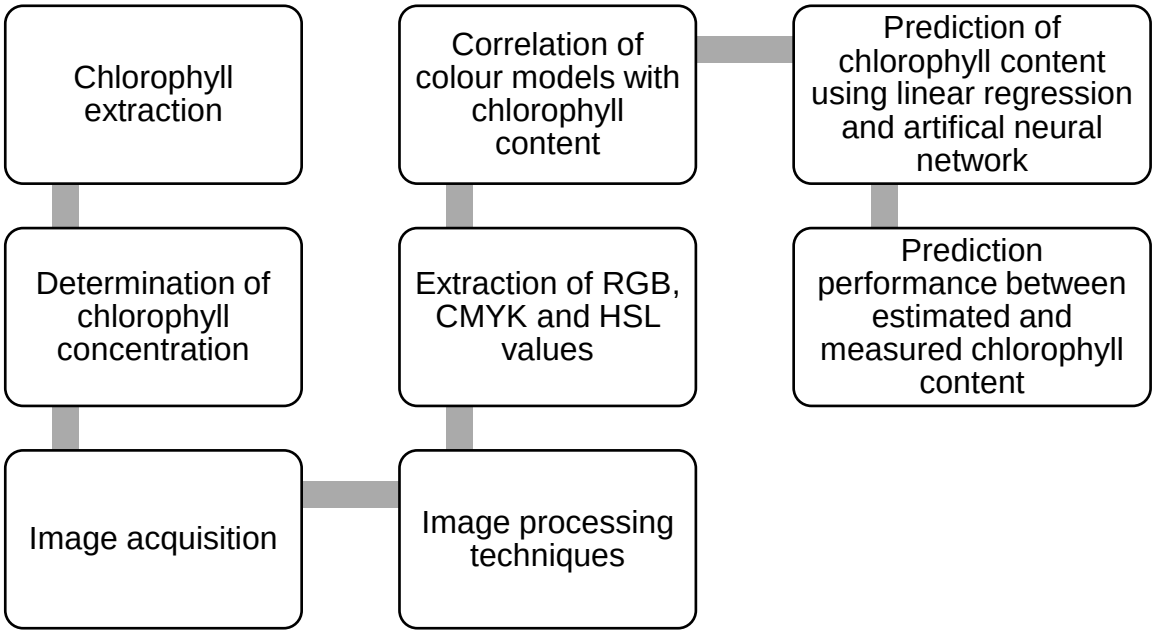

Figure 4.1. Experimental setup and procedure.

4.4 Result and discussion

4.4.1 Data acquisition

The suggested approach allowed for the simultaneous collection of data from microalgae, particularly in the producing of chlorophyll-based supplement products and monitoring of the microalgae growth. This

approach offered some benefits over the spectrophotometer in terms of time and cost. It is important to note that the homogeneity of the ambient lighting conditions during data collection may affect the result, for example twilight before and after daybreak when the blue spectral component predominates, as well as sunrise and sunset when red predominates [180, 241]. Next, due to same default conditions, the result of proposed method would not be affected by different brands of smartphones. In addition, the acquired images will not be impacted by the operating system of smartphone as the resolution of the images can be standardized. The smartphone imaging technique will prevent the blur and interference effects between the smartphone camera and the cuvette. The image processing software used in this study would not affect the results obtained as the software was used for image cropping and to obtain the index value. Although the suggested procedure was established in predicting the chlorophyll concentration, however, there was a concern on its direct application on other microalgae species because other species may not have similar colour index and chlorophyll values, making this a good starting point for further research. The species involved in this study was the microalgae culture collected from a power plant in Sweden, which consisted of the combination of strains, *Scenedesmus* sp. and *Desmodesmus* sp.

4.4.2 Linear regression

Linear regression is a straightforward machine learning tool for predicting an unknown parameter by matching the dependent and independent variables

using fitting line. SPSS software was used to perform linear regression on the final analysis data. Each colour component in the three colour models for various solvents type were considered as a single variable. It had been observed that the colour chromate and its chlorophyll content were linearly associated.

The interpretation of the correlation coefficient was determined using the Rule of Thumb [242]. The finding was demonstrated in **Table 4.1**. A significant correlation was observed between the chlorophyll concentration and primary colours of red-green-blue and cyan-magenta-yellow-black. A positive trend was observed with all the four-colour chromate of cyan-magenta-yellow-black colour model, and no correlation was observed between magenta colour and chlorophyll content in microalgae. In contrast to cyan-magenta-yellow-black colour model, a strong correlation with chlorophyll concentration was observed with red-green-blue colour model. Among the three primary colours in red-green-blue model, green colour chromate demonstrated the strongest correlation coefficient as compared to red and blue colour chromates. Next, the spectral information in hue-lightness-saturation model was also taken into consideration. A negative correlation was observed with hue and lightness parameters, demonstrating a weak and moderate association, respectively, whereas saturation parameter showed a moderate positive correlation. The correlation between hue-lightness-saturation and the chlorophyll concentration was considered weak. Thus, red-green-blue index was chosen for subsequent analysis. A

similar pattern of results was obtained in studies where the chlorophyll of photosynthetic organisms, including plants, was reliably predicted. Yadav, Ibaraki [243] used red-green-blue model to determine chlorophyll level of leaves of micropropagated potato plants and confirmed that red and green colour were negatively correlated with the chlorophyll concentration, but a positive association was seen with blue colour. Next, Riccardi, Mele [180] evaluated the applicability of red-green-blue to estimate the chlorophyll concentration in quinoa and amaranth leaves via the images captured using standard single-lens reflex camera at which the model offered greater association and prediction. The study by Zhang, Ge [181] estimated the chlorophyll content in leaves of sorghum using red-green-blue imaging at which green colour demonstrated good correlation with chlorophyll content as compared to red and blue colours. In short, red-green-blue model was more relevant in determining the chlorophyll concentration.

As compared to individual colour chromate in red-green-blue model, the colour index of red-green-blue model (sum of all red-green-blue colours) showed strongest regression correlation. In this case, one possible model with each extractant solvent was developed. The model expressed chlorophyll concentration as a function of red-green-blue colour index. The red-green-blue index with the acetone as extractant solvent was the best regression model with a correlation coefficient of 0.91. The predicted and targeted values of chlorophyll concentration were compared to validate the performance of the linear regression model. The data were qualitatively

close to the best fit line, with only a few points deviating from it. Moreover, in the comparison of extraction solvents, acetone showed the strongest correlation, and the data was significant (p-value < 0.05). This indicated that acetone was a good choice for extracting chlorophyll. A similar conclusion was reached by Franklin, Sathish [244] who also developed a multiple linear regression model with the R^2 of around 83.8% to estimate the chlorophyll-a concentration. It can be said that the linear regression model was very accurate and red-green-blue index was the most important feature in forecasting chlorophyll concentration.

4.4.3 Artificial neural network-based chlorophyll content predictor

In this study, a multilayer perceptron neural network was used to predict the concentration of chlorophyll in the images. Back propagation algorithm was used for system training. Using the artificial neural network-based chlorophyll content predictor, the predicted chlorophyll concentrations were compared to the results from the spectrophotometer measurement. Choosing the right technique is crucial to build a reliable prediction model for microalgae chlorophyll concentration. The estimation of microalgae chlorophyll concentration has been widely used with artificial neural networks technology. In the current study, 30% of the data set was used for testing, while the remaining 70% was chosen as training data. Using the error back propagation-training procedure, the estimated output value derived from the model was compared with the corresponding measured

values. By changing the RSME and model training time, the hidden layer's number of neurons was calculated.

Red-green-blue colour index with and without extraction solvent was selected as the input and compared using multilayer perceptron model in terms of prediction accuracy. The findings in **Table 4.2** demonstrated that multilayer perceptron model with the extraction solvents was robust to predict the chlorophyll concentration of microalgae with R^2 of 0.76 as compared to the model without solvents as the input (R^2 = 0.674). The accuracy between measured and predicted chlorophyll concentration was moderate high with average relative error of 48.6 %. However, the difference between these two models with and without extraction solvents were not significance. This showed that colour index obtained from image was sufficient to be fed into multilayer perceptron model regardless of solvent type as the higher the chlorophyll concentration, the deeper green colour indicated the higher levels of chlorophyll. For results comparison between the conventional spectrophotometry and artificial neural network method, residual analysis was conducted between the predicted and actual chlorophyll concentration. The residuals of the experimental and estimated chlorophyll concentration were dispersed at random around zero. This finding indicated how well the model fits to the data set. Moreover, multilayer perceptron model provided better accuracy (high R^2 value) for the prediction of chlorophyll concentration as compared to linear regression. This was because multilayer perceptron had numerous layers of neurons,

each with a threshold value and an activation function, providing incredibly precise results while there is no activation function or threshold in linear regression.

These findings were in accordance with the findings reported by previous literature studies. Odabas, Bajwa [245] applied image processing method to detect the chlorophyll concentration of St. John's wort leaf. To estimate the chlorophyll concentration, an artificial neural network model was created based on the red-green-blue of digital camera image with R of 0.99. Next, Odabas, Simsek [246] evaluated the nitrogen content of lettuce leaves through chlorophyll concentration using digital image processing. To forecast chlorophyll concentration, a multilayer perceptron neural network model was created based on the red-green-blue with mean square error of 0.006. Next, Mohan and Gupta [247] used smartphone to capture images of rice leaf under field conditions and modelling approaches to extract the chlorophyll level from the images. The created artificial neural network model with red-green-blue index, was found to be more effective in predicting the chlorophyll content than the linear regression model.

Testing and potential retraining of the neural network to forecast the make-up and dynamics of other extracted biomolecules from the microalgae should be part of the next-level research in this field. This will entail the precise distinction of other pigments, such as carotenoids, phycocyanin and astaxanthin, which were not here considered separately but rather just as part of the "other" fraction. In this work, the image processing treated these

pigments as the noise and subtracts them from the retrieved green pixels. Although the quality of the acquired image from the large field was dependent on several variables, including weather, time, shooting angle and camera, the effect of the variation among the acquired images on the artificial neural network prediction cannot be ignored in prediction works [234, 248]. It would also be interesting to compare different artificial neural network structures to the traditional multilayer perceptron neural networks used in this study. Overall, the microalgae chlorophyll concentration was validated and verified by two machine learning approaches, linear regression and multilayer perceptron. These models were only applicable in terms of determination of chlorophyll concentration in microalgae species. Using experimental data, multilayer perceptron model can be successfully trained to interpret the relationships between the input variables and correctly predict the output. The image processing technique can be considered as a valid approach for microalgae chlorophyll prediction. In addition, multilayer perceptron model can be applied as a prediction tool in the microalgae cultivation system by tracking the growth as well as determine the chlorophyll concentration in the manufacturing of bioproducts. This can help to save time and cost.

Table 4.1 Linear regression analysis of indices of various extraction solvents.

Solvents	Regression equation	R^2

		Max	Mean	Min
Acetone	X= 0.169(R) + 0.109(G) - 0.160(B) - 26.243	0.901	0.759	0.701
DMSO	X= 12.274 - 0.003(R) + 0.016(G) - 0.030(B)	0.767	0.580	0.600
Ethanol	X= 0.006(R) + 0.134(G) - 0.092(B) -9.715	0.732	0.552	0.548
Methanol	X= 11.990 - 0.0189(R) +0.026(G) - 0.021(B)	0.610	0.486	0.421

Table 4.2. Comparison of prediction accuracy between multilayer perceptron and linear regression models.

Criteria	Multilayer perceptron		Linear regression
	Training data	Testing data	
RMSE	2.01	2.36	3.1649
Relative error (%)	48.6	44.28	63.47
R^2	0.722	0.664	0.5833

4.5 Conclusion

In conclusion, this study revealed that regression and artificial neural network models offered the best prediction accuracy of chlorophyll concentration. When accounting for the variables, artificial neural network exhibited good outcomes on par with regression. Among the four extractant solvents, acetone was found to be excellent in extracting chlorophyll from microalgae. In the future, a little improvement in the statistical parameters of predictive models will be encouraging if it is projected on a big scale. The model can be further enhanced utilising deep neural network and create a multiplatform application for all users.

CRediT authorship contribution statement

Doris Ying Ying Tang: Conceptualization, Methodology, Writing – original draft, Project administration. **Kit Wayne Chew**, **Francesco G. Gentili**: Conceptualization, Methodology, Writing – review & editing. **Huong-Yong Ting**, **Yuk-Heng Sia**: Software, Visualization. **Young-Kwon Park**, **Fawzi Banat**, **Alvin B. Culaba**, **Zengling Ma**: Writing – review & editing. **Pau Loke Show**: Conceptualization, Methodology, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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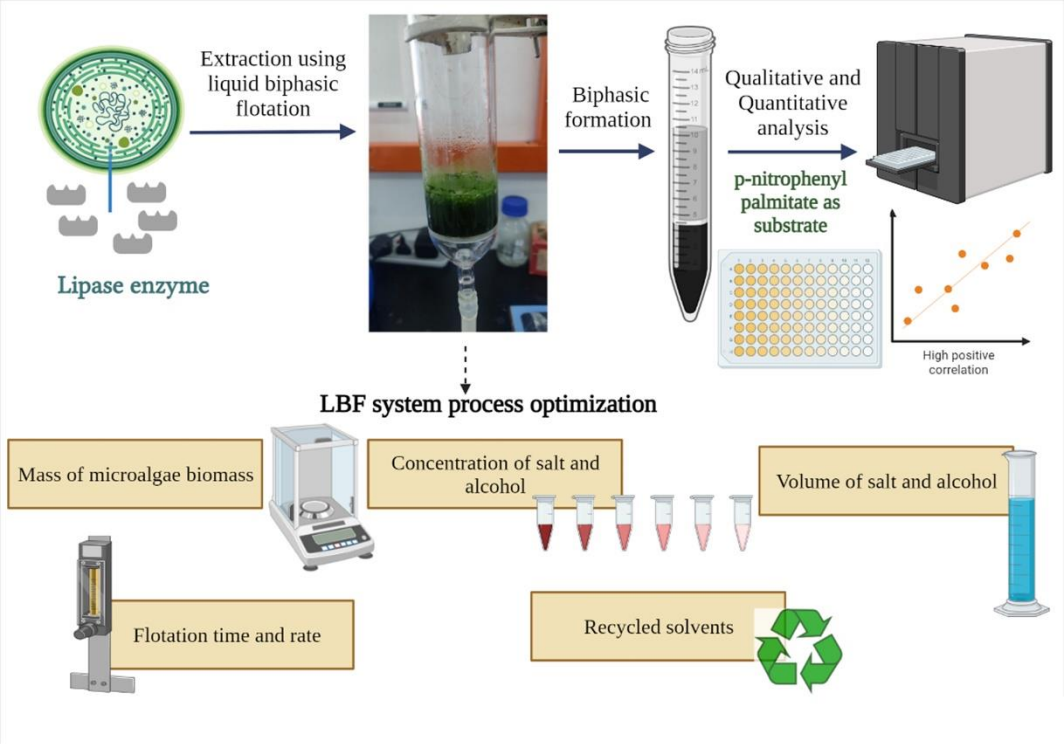
Data Availability Statements

All data generated during this study are included in this published article. The datasets analysed during the current study are available from the corresponding author on reasonable request.

CHAPTER 5 INVESTIGATION AND SCREENING OF MIXED MICROALGAE SPECIES FOR LIPASE PRODUCTION AND RECOVERY USING LIQUID BIPHASIC FLOTATION APPROACH



This chapter covers the application of liquid biphasic flotation system for the extraction of lipase enzyme from mixed microalgae biomass. Various operating conditions such as the solvent ratio, types of salt and alcohol, air flowrate, air flotation time, and addition of microalgae biomass powder are evaluated. This chapter consists of thesis-version of work published in the Journal of the Taiwan Institute of Chemical Engineers (https://doi.org/10.1016/j.jtice.2022.104646).

Investigation and Screening of Mixed Microalgae Species for Lipase Production and Recovery using Liquid Biphasic Flotation Approach

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5.1 Abstract

Background

Lipase is mainly extracted from animals, plants, fungi, and bacteria. The difficulty in extracting lipase from these organisms has led to the discovery of sustainable sources producing lipase. This study is the first to document the existence of lipase in the microalgae species from Umeå.

Methods

Lipase in microalgae was extracted using liquid biphasic flotation as the protein homogenate from the extracts was found to contain lipase activity. The lipase activity was evaluated using colorimetric method with *p*-nitrophenyl palmitate as the substrate. The types, volumes, and concentrations of solvents as well as flotation kinetics, and the biomass weight were optimized. The experiment was conducted at optimal temperature and pH 7.

Significant Findings

The optimized conditions for maximum lipase production were 400 mg of biomass, 10 min flotation time with flotation rate of 100 cc/min as well as 99.8% ethanol and 300 g/L ammonium sulphate concentration with volume ratio of 1: 0.83. Lipase recovery yield of the optimal LBF system was 70.3% with the separation efficiency of 82.0% and purification factor of 7.45. The findings revealed that the microalgae can be an ideal candidate for producing lipase and LBF serves as the platform for the development of large-scale extraction system.

Keywords: Microalgae lipase; Purification; Liquid biphasic flotation; Pharmaceutical; *P*-nitrophenyl

5.2 Introduction

Lipase enzymes are used in a variety of industries, including pulp, biodiesel production, food, pharmaceuticals, detergents, clothing, leather, cosmetics and wastewater treatment [249-251], because lipase can exhibit broad

substrate specificities, distinct physicochemical behaviour and excellent stability in the organic solvents [252]. Lipase is used to produce dairy products, detergents [253], pharmaceuticals (ibuprofen, naproxen), chemicals [254], pesticides, insecticides and oil [255]. The catalytic triad of most lipases is covered by a lid domain, and when this lid rotates around two hinge regions at the lipid-water interface, a significant hydrophobic patch forms around the catalytic triad, activating the lipase [256-258]. Lipase enzymes are effective in a variety of reactions [259-261], such as hydrolysis, esterification, transesterification [262] and C-C bond formation. Steric hindrance is defined as the spacer-arm insertion through enzyme and support. Low-molecular weight spacer arms typically consist of linear hydrocarbon chains with functions for any connection to the support and enzyme protein on both ends. There have been numerous studies on the use of different spacer arms to immobilise specific enzymes [263]. Enzyme immobilization methods [264-267] can be prepared via adsorption, cross-linking, covalent binding, encapsulation and entrapment [268]. Nowadays, enzyme immobilization has proven to be effective for commercial applications [254, 269-271] due to lower cost, greater stability, and the potential to be simply removed from the reaction mixture, allowing for the isolation of the pure product [272].

Lipase is widely distributed in plants [249], mammals, bacteria [273, 274], yeast [275] and fungi [276]. Among the organisms, microbial lipases, for example *Pseudomonas fluorescens*, *Staphylococcus aureus*,

Burkholderia pseudomalle, *Bacillus pumilus*, *Bacillus subtilis* and etc., are currently receiving a lot of attention in biotechnology field and industries. There are less studies about the isolation and characterization of lipases from the native microalgae sources, making microalgae the intriguing source of lipases. Microalgae thus represents the enzyme resource that has not been extensively and commercially used. Discovery of lipase from *Spirulina platensis*, a filamentous cyanobacterium, was first described in 2009, which had the characteristics of 45 kDa molecular weight and isoelectric point of 5.9 [6]. Besides, Godet, Hérault [70] identified a novel gene from the microalgae *Isochrysis galbana* that coded for a 49 kDa lipase of 457 amino acids which was resembled lipases. Nalder [277] characterized lipase from the microalgae *Chaetoceros calcitrans*, *Isochrysis sp.* and *Pavlova lutheri*. The study by Yong, Lim [7] used *Botryococcus sudeticus* UTEX 2629 to synthesise extracellular lipase which demonstrated good thermal stability and 90% similarity to *S*. *mobaraensis* esterase. The potential for lipase to be used in various sectors can be seen by the fact that their activities at the optimum conditions that were 50 °C and pH 10 were nearly two and seven times higher than their lowest activities, respectively. Savvidou, Sotiroudis [10] found a thermostable lipolytic enzyme from *Nannochloropsis oceanica* that exhibited 2.5-fold stimulation after heating to 100 °C for 60 minutes and had an optimum pH of 7.0.

Liquid biphasic flotation (LBF) is a bioseparation technique that incorporates bubbling action into the combination system liquid biphasic

system and adsorptive bubble flotation to extract and purify the biomolecules from the organisms, such as protein, carbohydrate, enzyme, antibiotics, antioxidants and other biomolecules. The LBF system works by allowing the biomolecules from the bottom aqueous phase containing surface-active sites, to be adsorbed preferentially onto the surfaces of ascending gas bubbles and be collected in the top aqueous phase [278]. Previously, salt or solvent precipitations, membrane processes and chromatography methods were used to extract and purify the lipase from various sources. However, some of these conventional methods are time-consuming, low yield and expensive [279]. Therefore, a novel technique called LBF system is applied in this study to extract and purify lipase from the microalgae. The proposed system in this present study offers a lot advantages, including short processing time, high biocompatibility, simple, non-toxicity and recyclability, which support its potential as an effective platform for lipase production in industrial scale [280-282].

The lipase-producing potential of microalgae, such as *Spirulina platensis*, *Isochrysis galbana*, *Chaetoceros calcitrans*, *Isochrysis* sp., *Pavlova lutheri*, *Botryococcus sudeticus* and *Nannochloropsis oceanica* have been explored in prior studies. This paper addresses the potential of the microalgae culture consists of *Scenedesmus* sp. and *Desmodesmus* sp. strains, which is currently lacking in the scientific literature. Hence, in this study, we examine the lipase-producing potential of the microalgae species that collected from raceway pond at Umeå Energy. The microalgae species

is found to contain mixed strains of *Desmodesmus* sp. and *Scenedesmus* sp. The recovery of lipase from microalgae using alcohol/salt LBF has not yet been studied extensively. Different parameters for the LBF are optimized to evaluate lipase yield, selectivity, purification factor and separation efficiency. The variables analysed are types and concentration of alcohols and salts, volume of solvents, flotation kinetics, and the microalgae biomass weight. This work is contributed by producing the breakthrough finding that uses LBF to recover lipase from microalgae economically and can be used at an industrial scale.

5.3 Methodology

5.3.1 Materials

Chemicals utilised in the LBF system were acquired from R&M Chemicals. The lipase standard and 4-Nitrophenyl palmitate (*p*-NPP) were applied in the enzyme assay and purchased from Merck Malaysia. Freeze dried *Desmodesmus* sp. and *Scenedesmus* sp. microalgae powder, was provided by Swedish University of Agricultural Sciences (a generous gift from Dr. Francesco Gentili). The microalgae was cultivated at Umeå, northern Sweden (63°86′ N) at Umeå Energy combined heat and power plant (CHP-plant) using municipal untreated wastewater influent [188].

5.3.2 Apparatus

The LBF equipment used in this study is manufactured by Donewell Resources Sdn. Bhd (Selangor, Malaysia). It is made of glass and divided

into two types, which were small scale (50 mL) and large scale (300 mL). G4 sintered disk with pore size of 5-15 µm was used to construct the base of the column of LBF system. The base was connected to air pump (Rocker Oil Free Vaccum Pump, Rocker 300) attached to Dwyer flowmeter (model VFA-34, range 20-200 cc/min) [283].

5.3.3 Methodology

5.3.3.1 Extraction of lipase using LBF system

Extraction of lipase from microalgae biomass was conducted using LBF system in different conditions or parameters via one-factor-at-a-time method. Prior to extraction, the wet microalgae slurry was pre-treated to disrupt the cell wall to release desired biomolecules. The LBF system was started with the addition of salt solution, followed by alcohol for two-phase formation to extract lipase. Various LBF parameters as mentioned in **Table 5.1** were optimized for maximum extraction of lipase. The initial settings of the LBF comprised of 10 mg of dried microalgae powder, 20 mL of each salt and alcohol solvents, 100% (w/w) of ethanol, and 300 g/L $(NH_4)_2SO_4$. The initial flotation time and rate for the separation process was set at 10 minutes and flowrate of 50 cc/min. The experiment was conducted at room temperature. For the recycling studies, top alcohol phase was recycled first followed by bottom salt solution to ascertain which phase is suitable for recycling purpose.

Table 5.1 Operating parameters.

Parameters	Unit	Initial setting	Other settings
Mass of dried microalgae biomass	mg	50	100, 200, 300, 400
Type of food-grade alcohol	-	Ethanol	Methanol, 2-propanol, 1-propanol
Type of salt		Ammonium sulphate	Magnesium sulphate, di-potassium hydrogen phosphate, sodium carbonate
Concentration of alcohol	% (w/w)	99.8%	50, 60,70,80,90
Concentration of salt solution	g/L (w/v)	300	150,200,350
Volume of alcohol	mL	20	10,15,25
Volume of salt	mL	20	10,15,25
Flotation time	min	10	5,15,20,25

Flotation rate	Cc/min	100	50, 150, 200
Recycled solvent	-	-	Recycled ethanol and ammonium sulphate

5.3.3.2 Analytical methods

The quantification of total protein in the samples was evaluated using Thermo Scientific Pierce BCA Protein Assay Kit. Next, the activity of lipase was evaluated using *p*-nitrophenylpalmitate (*p*NPP) as the substrate. The reaction mixture was comprised of 0.9 mL substrate solution (100 mM *p*-nitrophenyl diluted in ethanol), 0.5 M buffer (0.1 mL) and diluted enzyme (0.1 mL), followed by incubation at 37 °C for 5 minutes. The lipase activity was measured at room temperature using a UV–vis spectrophotometer (model UV-1800, Shimadzu, Japan) and the absorbance at 410 nm was recorded. The amount of *p*-NP (*p*-nitrophenyl) emitted from the *p*-NPP hydrolysis was determined using the standard curve of *p*-NP. The volume of lipase required to release 1 µmol of *p*-NP per min was equal to one unit (U) of lipase activity. From the standard curve, Eq. (1) was derived for the determination of lipase activity (µmole/min/mL).

$$\text{Lipase activity} = \frac{Absorbance\ at\ 410nm}{0.1767} \quad \text{..... Equation 5.1}$$

5.3.3.3 Calculation of partition coefficient, yield of lipase, purification factor, selectivity and extraction efficiency

i. Partition coefficient (K):

$$K = \frac{L_T}{L_B} \text{..... Equation 5.2}$$

- L_T: Lipase activity in top phase
- L_B: Lipase activity in bottom phase

ii. Volume ratio (V_R):

$$V_R = \frac{V_T}{V_B} \text{.....Equation 5.3}$$

- V_T: Volume of top phase
- V_B: Volumes of bottom phase

iii. Yield of lipase:

$$Y_T(\%) = \frac{100}{1+(\frac{1}{V_R}\times K)} \text{.....Equation 5.4}$$

- V_R: Volume ratio
- K: Partition coefficient

iv. Extraction efficiency (E_L):

$$E_L = \frac{K\times V_R}{1+ KV_R} \text{ ..... Equation 5.5}$$

- V_R: Volume ratio
- K: Partition coefficient

v. Purification factor (P_{FT}):

$$P_{FT} = \frac{SA\ of\ top\ phase}{SA\ of\ crude\ feedstock} \text{.....Equation 5.6}$$

- SA: Specific activity (ratio of enzyme activity and protein concentration)

vi. Selectivity (S):

$$S = \frac{K_e}{K_p} = \frac{E_T}{E_B} \times \frac{P_B}{P_T} \text{..... Equation 5.7}$$

- P_T: Protein concentration at top phase
- P_B: Protein concentration at bottom phase
- E_T: Lipase concentrations at top phase
- E_B: Lipase concentrations at bottom phase

5.3.4 Statistical analysis

For each condition, three independent runs were caried out. The findings were presented as the mean ± standard error of mean deviation. To analyse the variations between the means of various conditions of a single factor, one-way analysis of variance was performed. Significance test was fixed at an alpha level of 0.05. The statistical analysis was performed using SPSS Statistics software. The graphs with error bars in this study were plotted using SigmaPlot v12.0.

5.4 Result and Discussion

In this study, different *p*-nitrophenyl, that were *p*-nitrophenol, *p*-nitrophenyl laurate and *p*-nitrophenyl palmitate, were compared in the preparation of standard curve. Among these *p*-nitrophenyl substrates, *p*-nitrophenyl palmitate demonstrated satisfactory result because its long-chain palmityl group had the ability to distinguish between lipase activity and

esterase activity [284], therefore used for assessing the lipase activity of the samples. Most of the enzymes, including lipase, are proteins, so they can be easily denatured by heat or high temperature. Besides, optimum pH for most of the enzymes are ranging from pH 7 to pH 9. Therefore, the sample preparation, selection of extraction technique and conditions are essential for the stability of lipase so that the lipase would not degrade, denature or lose its activity during the extraction process. LBF is a suitable extraction method to extract lipase from the microalgae because it does not involve heat and the solvents used are suitable to maintain lipase activity. The experiment is conducted at optimal temperature (room temperature) and optimal pH (pH 7). As compared to the bottom phase of the system, the top phase had the highest lipase activity. There were a few parameters applied to optimize the extraction of lipase from the microalgae, such as the mass of biomass, use of alcohol and salt, flotation rate and time as well as the recyclability of the solvents.

5.4.1 Mass of microalgae biomass

The biomass weight in the system will influence the partitioning behaviour of the proteins and thus affecting its yield. In this study, the examined mass of microalgae ranged from 50 mg to 400 mg (**Figure 5.1**). In 50 mg microalgae biomass, the lipase yield was the highest with the lowest extraction efficiency and partition coefficient because there was more space available for the lipase enzyme and unwanted proteins to move to the top phase, result in higher yield with decreased purity. On the other way,

400 mg microalgae biomass demonstrated the highest extraction efficiency and partition coefficient with moderate lipase yield. The purification factor of 400 mg was the highest, which was 4.21. The study by Chia, Chew [285] and Chew, Chia [280] reported that lower biomass concentration was unfavourable as it could result in lower recovery of the desired biomolecules which will reduce the effectiveness of the purification process. On the contrary, the lipase separation may be impacted by the viscosity of solution and it was highly possible for the presence of impurities or contaminants in the system due to high biomass concentration. Hence, by looking at the satisfactory performance, the microalgae biomass of 400 mg was chosen as the best condition for the subsequent LBF study of other parameters.

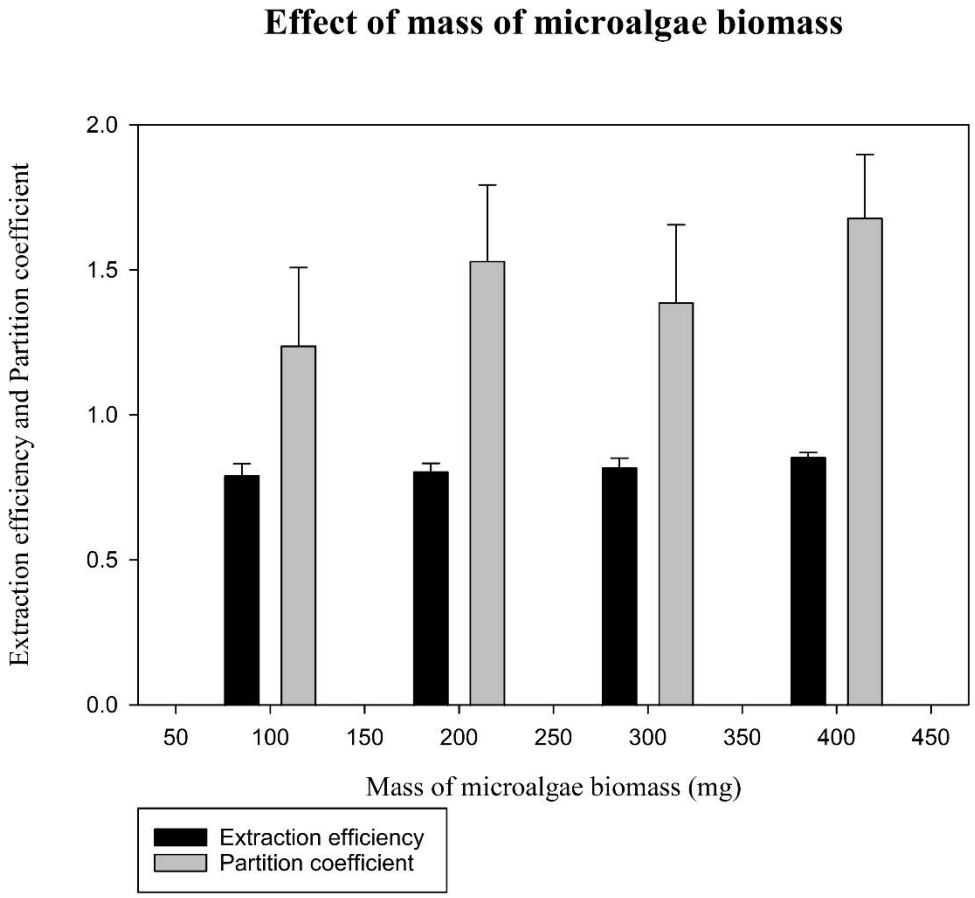


Figure 5.1 Effect of mass of microalgae biomass on extraction efficiency and partition coefficient. The LBF conditions were 300 g/L ammonium sulphate, absolute ethanol and flowrate of 100 cc/min in 10 min. The result findings were presented as means of three duplicates readings and statistically significant.

5.4.2 Type of food grade alcohol and salt

The selection of solvents for the top phase and bottom phase depended on the polarities of the solvents and targeted bioactive compounds [286]. In this study, different types of salt and alcohol were investigated for their ability to separate lipase. One of the phase-forming elements of LBF, which

was salt, had a major impact on the liquid-liquid phase equilibria. When salt was added to a solution, the surface tension of water rose, which increased the hydrophobic interaction between proteins and water [274, 287]. Due to the salting-out effect, the desired biocompounds will accumulate at the hydrophilic solvent phase. Four different types of salt which were ammonium sulphate, magnesium sulphate, di-potassium hydrogen phosphate and sodium carbonate were investigated. The findings as shown in **Table 5.2** demonstrated that ammonium sulphate obtained the highest lipase yield among four salt types. Throughout the experiment, the development of salt crystals at the bottom phase, except ammonium sulphate, suggested that the two liquid phases in LBF system were not in equilibrium, making the system less ideal for the separation procedure. In the purification of proteins and enzymes, ammonium sulphate was frequently used to precipitate proteins because sulphate ions in ammonium sulphate tended to interact with water, which resulted in a high protein yield and good hydration capacity. On top of that, ammonium sulphate, also known as traditional kosmotropic salt, was commonly used for salting out of protein from aqueous solution and encouraged hydrophobic interactions to increase protein stability [288]. However, a large portion of undesirable proteins were distributed at the top phase, reducing the purity of desired biomolecules [280, 285].

Next, various types of hydrophilic organic solvents were explored in LBF system for the top phase. The alcohol used in this study were food grade

status and did not involve the use of hazardous solvents, for example hexane, benzene, chloroform, ethyl acetate, dimethyl sulfoxide or hydrochloric acid. Alcohols with low concentration, low polarity and longer carbon chain aided in formation of the two-phase system [289]. Overall, ethanol demonstrated satisfactory performance in terms of separation efficiency, recovery yield and selectivity. Based on the results, biphasic formation cannot occur when methanol and n-butanol reacted with the tested salts. This was supported by the study by Leong, Ooi [290] and Phong, Show [291] that two-phase formation cannot be observed for methanol-salt based LBF. This was brought on by low concentration of salt and the rapid evaporation rate of methanol which caused a quick loss of the solvent from the top phase [290]. Khoo, Chew [292] also reported that ethanol and 2-propanol were suitable to be used as alcohol-based phase component in LBF system to extract astaxanthin from *H. pluvialis*. However, in this study, 2-propanol showed unsatisfactory result as compared to ethanol. Hence, ethanol and ammonium sulphate were selected as the best combination for the following experiments as this combination at high concentration will form two phases due to their hydrophobic and hydrophilic interaction.

Table 5.2 Effect of type of alcohol and salt in lipase yield, extraction efficiency, selectivity and partition coefficient of lipase enzyme. The LBF conditions were effect of varied alcohol and salt types, 400 mg of microalgae biomass, alcohol concentration of 100%, salt concentration of 300 g/L, flowrate of 100 cc/min in 10 min. The result findings were presented as means of three duplicate readings and statistically significant.

Type of alcohol	Type of salt	Biphasic formation in LBF	Lipase yield in top phase (%)	Extraction efficiency	Partition coefficient	Selectivity	Purification factor
Ethanol	$(NH_4)_2SO_4$	Yes	70.1	0.7557 ±0.026	1.2393 ±0.15	0.1043 ±0.013	4.56
	$MgSO_4$	Yes with the formation of salt crystal	20.3	0.4318 ±0.02	7.1628 ±0.80	5.4520 ±0.20	3.32

	K_2HPO_4	Yes with the formation of salt crystal	15.2	0.4548 ±0.02	7.1506 ±0.90	4.1238 ±0.52	2.05
	Na_2CO_3	Yes with the formation of salt crystal	14.6	0.4988 ±0.02	6.1200 ±0.56	3.1485 ±0.89	1.68
Methanol	$MgSO_4$, K_2HPO_4, Na_2CO_3	No	NA	NA	NA	NA	NA
1-propanol	$(NH_4)_2SO_4$	Yes with the formation of precipitate	14.9	0.8318 ±0.03	4.1511 ±0.76	3.7253 ±0.66	1.89

	$MgSO_4$, K_2HPO_4, Na_2CO_3	No	NA	NA	NA	NA	NA
	$(NH_4)_2SO_4$	Yes	18.5	0.9143 ± 0.054	8.107 ±0.316	5.9048 ±0.23	2.23
2-propanol	$MgSO_4$, K_2HPO_4, Na_2CO_3	No	NA	NA	NA	NA	NA
n-butanol	$(NH_4)_2SO_4$	Yes, with the formation of crystal	60.0	0.5027 ±0.080	0.9977 ±0.43	2.1786 ±0.94	3.46

$MgSO_4$, K_2HPO_4, Na_2CO_3	No	NA	NA	NA	NA	NA

5.4.3 Alcohol and salt concentration

LBF can be impacted by the variations in the alcohol and salt concentration. The variation in solute partitioning will result in the change of physical properties of the system, for example surface tension, density and viscosity [280, 285, 292]. Selection of appropriate of concentration of salt and alcohol was essential for the optimal atmosphere to ensure survivability of lipase. Therefore, the separation efficiency of lipase was investigated by varying the alcohol concentrations (99.8%, 80%, 50%) and salt concentration ranging from 200 g/L to 350 g/L. The impact of alcohol concentration on the lipase extraction and partition efficiency was illustrated in **Figure 5.2**. Diluting the ethanol concentration will decrease the extraction efficiency and partition efficiency and thus result in low purity of lipase in the top phase. Based on the results, alcohol concentration of 99.8% was chosen because reduced concentration of alcohol will affect the efficiency and performance of LBF system.

The salt concentration was altered and at the same time, the ethanol concentration was held constant. The influence of ammonium sulphate on the lipase extraction and partition efficiency were demonstrated in **Figure 5.3**. When the salt concentration increased to 350 g/L, partition efficiency reduced but the extraction efficiency increased. Lipase yield was the highest at 350 g/L of salt concentration which was 81.09% with purification factor of 3.45 because of the precipitation of the lipase enzyme at high salt concentration. This will increase the free volume

indirectly and caused partitioning of desired compounds and contaminant proteins to the top phase. When combined with the flotation effect by air bubbles, more contaminants would be transported to the top phase which could reduce the purity of lipase. Too high salt concentration will also affect the enzyme active site and their reactivity [274]. Nevertheless, the high volume ratio in the typical LBF system needed high salt concentration to support an immiscible two-phase via the salting-out effect [278]. Hence, 300 g/L of salt content instead of 350g/L was chosen throughout the study.

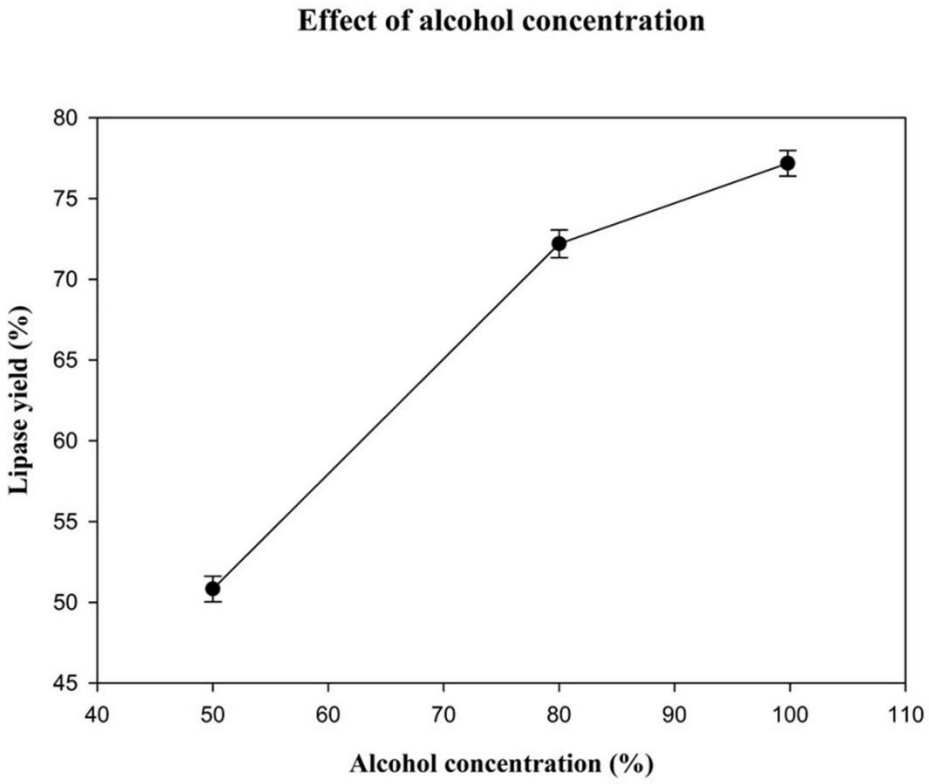


Figure 5.2. Effect of alcohol concentration on lipase yield, extraction efficiency and partition coefficient. The LBF conditions were 400 mg of microalgae biomass, 300 g/L of ammonium sulphate and air

flowrate of 100 cc/min in 10 min. The results were statistically significant.

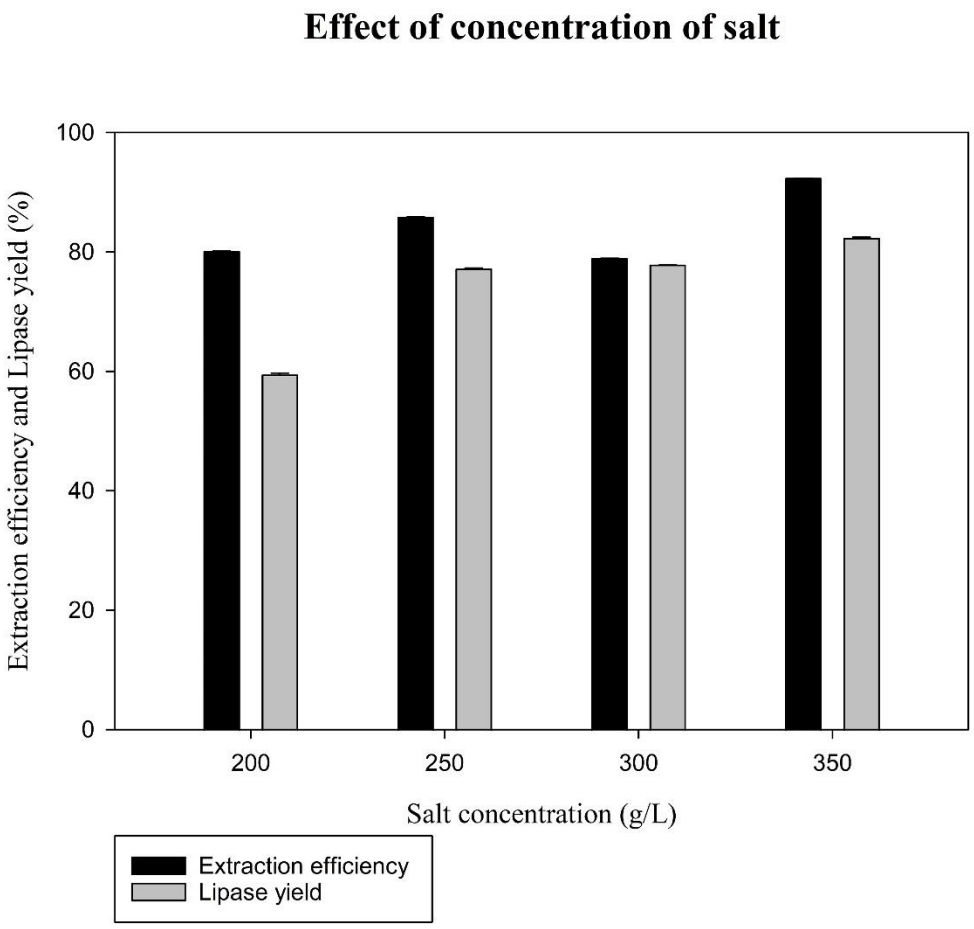


Figure 5.3. Effect of salt concentration on lipase yield and extraction efficiency. The LBF conditions were 400 mg of microalgae biomass, absolute ethanol, air flowrate of 100 cc/min and flotation time of 10 min. The result findings were presented as means of three duplicate readings and statistically significant.

5.4.4 Volume of solvents

In a liquid biphasic system, changes of the volume of solvents in top and bottom phase or volume ratio can alter the yield, extraction efficiency, partition efficiency and purity of the recovered biomolecules. The volume ratio increased when the volume of aqueous solution decreased. The volume ratio of the system was changed from 1: 0.3 to 1: 1.2 to observe the lipase yield in each phase (**Figure 5.4**). The lipase yield increased but the purity reduced as the volume ratio increased. Greater volume ratio will attract more lipase due to high volume of alcohol. The volume ratio of 1:0.83 produced the excellent extraction efficiency, which showed a 30% improvement over the initial volume ratio of 1:1. Lowest partition and extraction efficiency as well as lipase yield were recorded by the lowest volume ratio. The rise in volume ratio will result in the increase of top phase volume, which allowed for the condensation of desired molecules (lipase enzyme) and the contaminant proteins. The lipase yield would increase with reduced purity due to the presence of proteins at the top phase. Conversely, lowering the volume ratio will reduce the volume of top phase and increase total salt concentration. This result in smaller amount of free volume of the lipase and proteins. The volume of bottom salt aqueous phase was always greater than alcohol top phase [293]. The volume ratio of 1:0.83 produced satisfactory result, so this volume ratio was used for further studies.

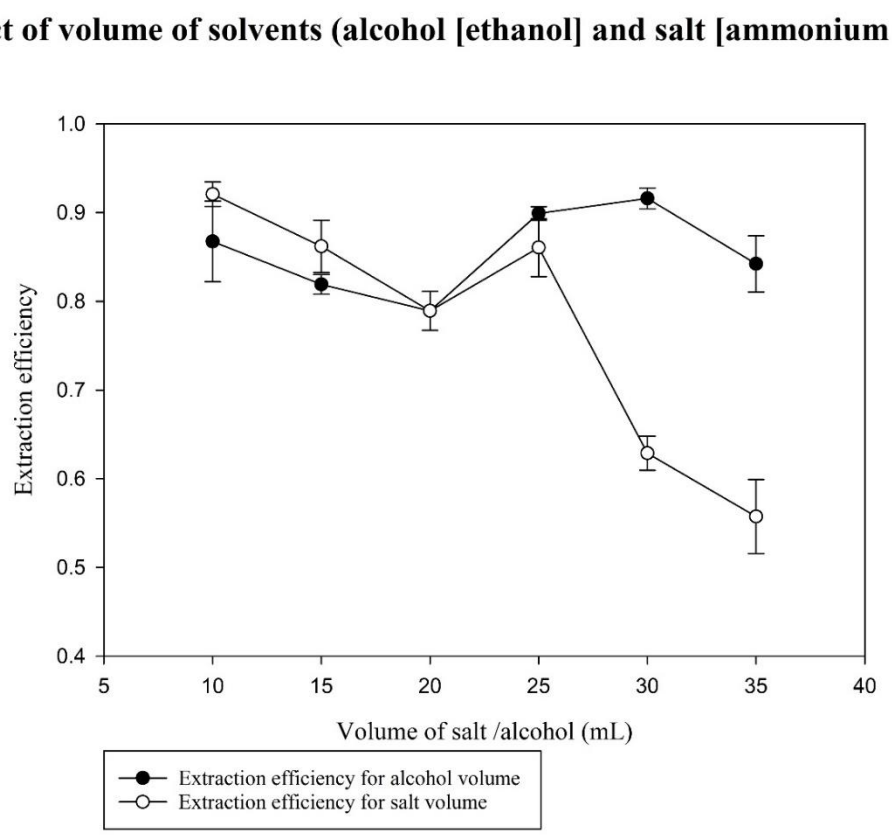


Figure 5.4. Effect of solvents volume on extraction efficiency. The LBF conditions were 400 mg of microalgae biomass, ethanol concentration of 100%, and ammonium sulphate concentration of 300 g/L, air flowrate of 100 cc/min in 10 min. The result findings were presented as means of three duplicate readings and statistically significant.

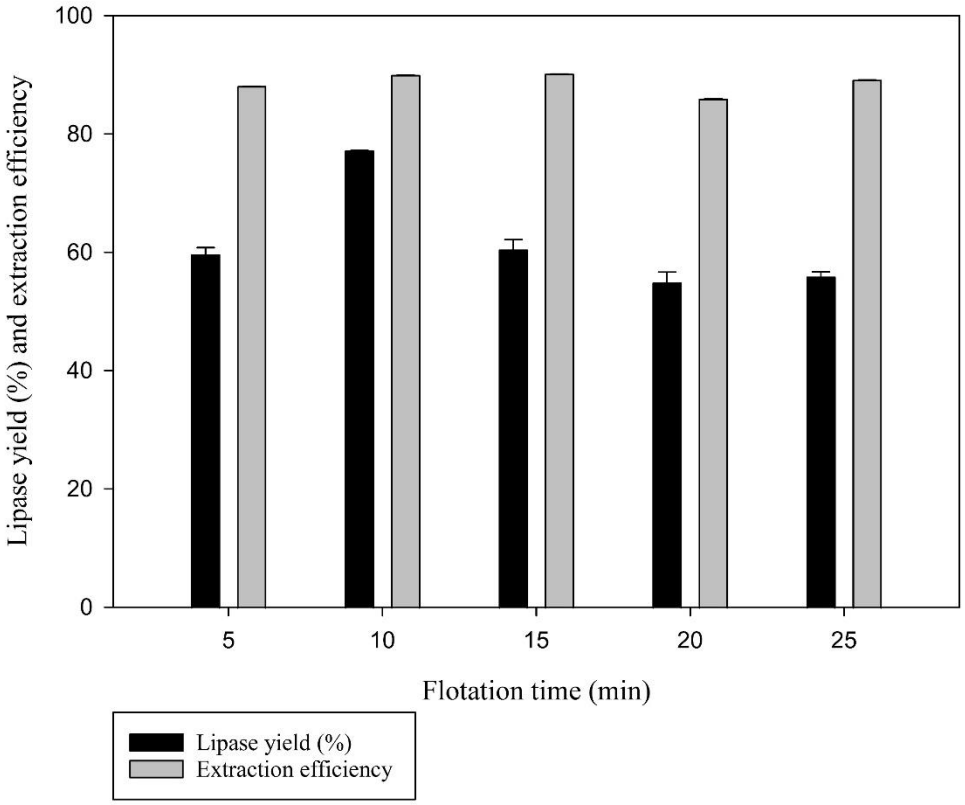
5.4.5 Flotation time and rate

Air flotation time played a significant role in the LBF system [274]. It was important to consider the size of air bubbles in relation to the interfacial area for the mass transfer of gas-liquid. To reduce the interfacial tension, very small bubbles will combine to form larger bubbles. In this

study, the flotation rate was set to be around 50-200 cc/min. Concurrently, different flotation time ranging from 5 minutes to 20 minutes were assessed to partition lipase in LBF system. According to **Figure 5.5**, the extraction efficiency was highest at 150 cc/min with 15 min. Despite that 150 cc/min demonstrated highest extraction efficiency, the lipase yield was highest at 100 cc/min. Beyond air flowrate of 100 cc/min, the lipase yield, purity and selectivity decreased. Increased flotation rates will result in high turbulence mixing, which caused the lipase to re-dissolve at the bottom. Furthermore, an accumulation of the top phase of the system will happen when flow rate was high and large number of gas bubbles could not burst at the same time [274, 294, 295]. Therefore, the appropriate flotation rate for further research was determined to be 100 cc/min.

In terms of flotation time, for the first 10 minutes, the yield of lipase rose but decreased after 10 min despite flotation time of 15 min showed the highest extraction efficiency and purification factor of 4.4. Increasing flotation time will encourage the partitioning of lipase to the top phase, but at the same time, also partitioned contaminated proteins to the top phase. Therefore, higher flotation times produced biomolecules with high yields with reduced purity. 10 minutes of flotation time was chosen as the appropriate flotation time because it produced the highest purity and comparable lipase yield when compared to the initial setting of 5 min. Further flotation after 10 minutes of flotation time was shown to be unnecessary.

Effect of flotation time



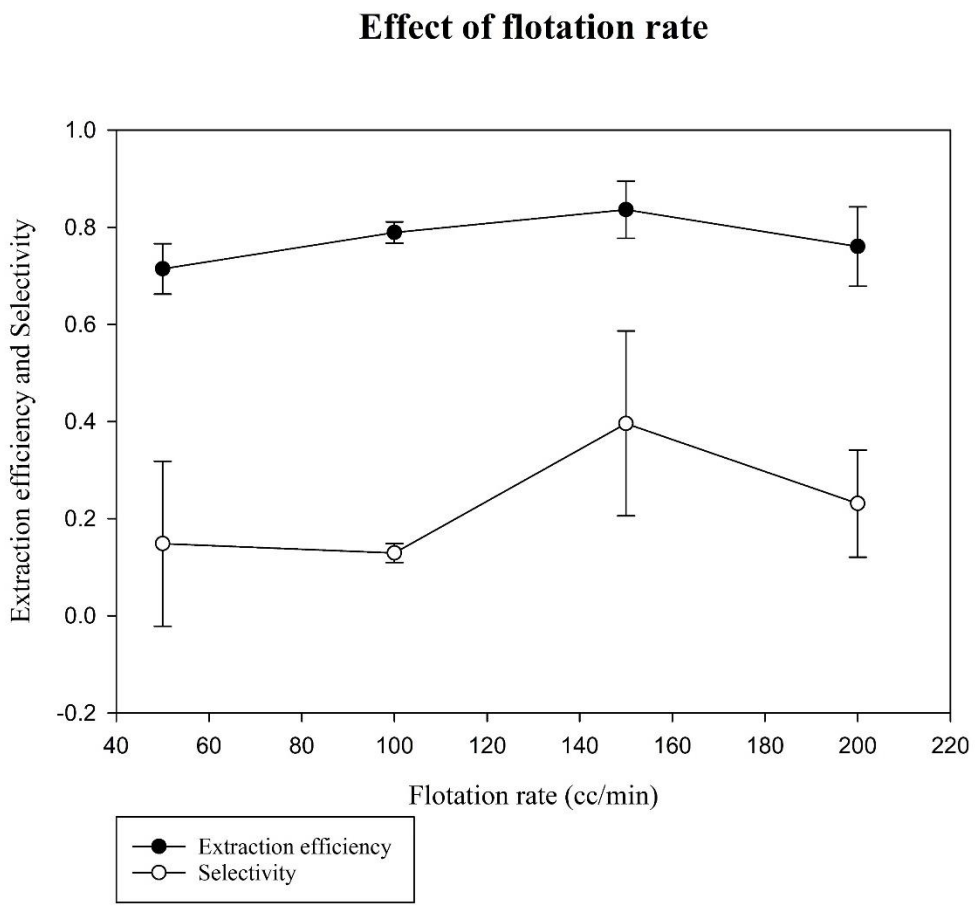


Figure 5.5. Effect of flotation kinetics on lipase yield, selectivity and extraction efficiency. The LBF conditions were 400 mg of microalgae biomass, ethanol concentration of 100%, and 300 g/L ammonium sulphate with volume ratio of 1: 0.83. The result findings were presented as means of three duplicate readings and statistically significant.

5.4.6 Effect of recycled solvent

Figure 5.6 demonstrated the influence of recycling alcohol (ethanol) and salt (ammonium sulphate) in the laboratory scale. After the extraction process, alcohol in the top phase was separated using rotary evaporator whereas the salt in bottom phase was separated by adding in methanol followed by filtration and rotary evaporation. The solvents were then used for recycling studies. The benefits of solvent recycling can reduce the cost in the aspect of chemical purchasing, minimize the waste disposal and reduce the environmental impact. According to the findings (**Table 5.3**), recycled alcohol from the top phase showed high recovery yield, protein recovery and extraction efficiency. However, in the second ethanol recycling phase of LBF, the separation efficiency of lipase decreased significantly due to the presence and saturation of contaminants or other impurities at the aqueous top phase which reduced the effectiveness of separation. Nevertheless, the performance of recycled salt at bottom phase were unsatisfactory due to high water retention at the salt phase, affecting the separation and extraction efficiency. The salting out action and protein mass transfer may be complicated by the high volume of salt solution. The earlier investigation on the impact of concentration and volume of salt solution in LBF provided additional support for this, showing that the yield, separation efficiency and partition efficiency decreased as the volume of ammonium sulphate solution increased. In addition, the reusability of enzyme was another issue for future research to explore for the continuous application of enzymes in various sectors to retain enzyme

activity and reduce production costs for industrial application [250, 296]. In short, the reuse of solvents in the LBF system is a novel, affordable, and efficient way to separate biomolecules, proving the value of using these phase components for biomolecule extraction in industrial settings.

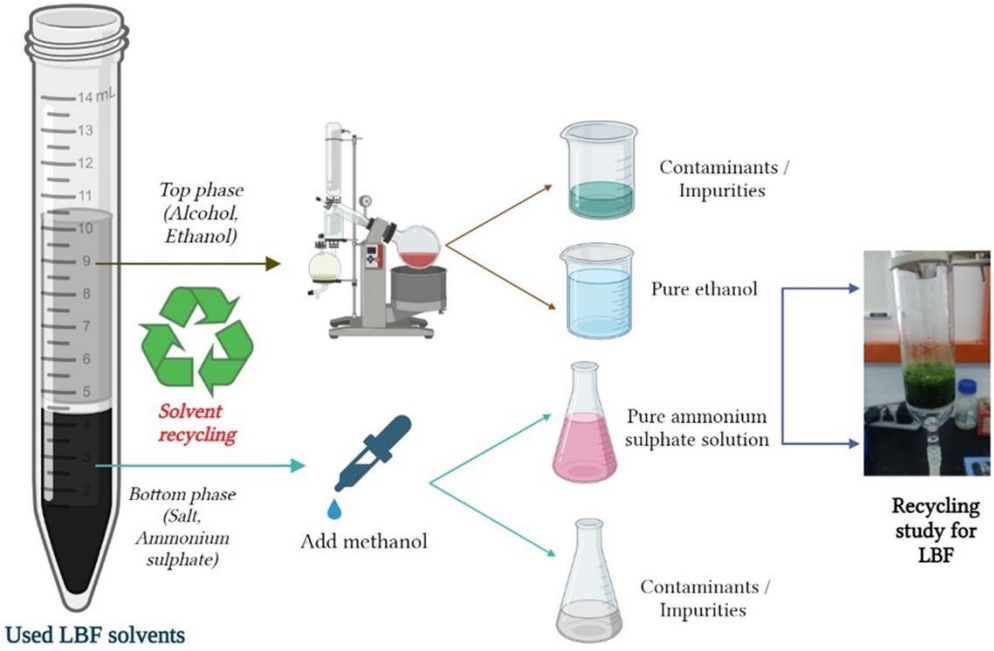


Figure 5.6. Recycling study of the phase components of LBF.

Table 5.3. Effect of recycled ethanol (top phase) on extraction efficiency. The result findings were presented as means of three duplicate readings and statistically significant.

	Recycled alcohol phase	First extraction

Partition coefficient	2.13 ± 1.23	1.28 ± 0.27
Separation efficiency (%)	77.5 ± 0.64	73.25 ± 0.89
Lipase yield (%)	70.7	66.3
Purification factor	5.76	5.32

5.4.7 Optimized LBF System and Scale-up of the system

Based on the results of experiments on single factor, 400 mg microalgae biomass, 99.8% ethanol and 300 g/L ammonium sulphate combination, volume ratio of 1:0.83, neutral pH, air flotation of 100 cc/min and 10 min were discovered as the optimal LBF conditions. It is crucial to comprehend how the factors interact with each other in order to evaluate the process as a whole. Three verification tests with the optimized conditions were conducted and produced mean lipase yield of 70.3% (**Table 5.4**). The separation efficiency and lipase yield of the optimized system as compared to control LBF system was increased by 10.5% and 16%, respectively. The result was compared with the study by Sankaran, Show [274], that extracted lipase from the bacteria, *Burkholderia cepacia* and produced lipase yield of 95.7% and 93.8% in laboratory scale and upscaling of the flotation system, respectively. When compared to *Burkholderia cepacia*'s lipase yield, the microalgae involved in this study

produced a low amount of lipase. Therefore, the comparison of different type of microalgae species in the production of enzyme lipase that is comparable to the commercial lipase-producing microorganisms and the way to enhance the extraction efficiency of lipase enzyme from the microalgae can be carried out in the future.

The optimized system in this study was then performed in large scale with total volume of 300 mL, which comprised of equal amount of alcohol and salt. The scale-up of LBF system was aimed to evaluate the efficiency of this extraction system for prospective use at pilot scale. The findings revealed that large-scale LBF system demonstrated low separation efficiency than small-scale, but lipase yield and protein recovery were slightly higher. In summary, the scaling-up of LBF system from the small scale to the pilot scale with identical optimized parameters was competent and can generate better results through the modification of the process parameters to increase the yield as well as to assess its feasibility in various sectors in the future. This scaling up method demonstrated the promise of LBF as a different strategy for the recovery of lipase from the microalgae.

Future LBF studies can employ an air compressor to deliver greater and more precise flowrates as well as the use of nanobubbles to enhance extraction efficiency and yield of protein recovery. Further research on the interaction factors, such as lipase yield with the solvents type, pH and temperature of the system, could have a high likelihood of boosting the

enzyme production. Additionally, the extractant solvents used as the top and bottom phase can be changed to other chemicals that are green chemicals or food grade to minimize the environmental impacts. The LBF system also can incorporated with assisted treatment techniques such as sonication [297] or electroporation of cell membrane [298] to increase the yield of desired biomolecules. Lastly, future research should consider the effects of diffusion limitations more carefully, for example through surface modification of LBF system for enzyme immobilization to overcome diffusion limitations.

Table 5.4. Comparison study of small scale and large-scale liquid biphasic flotation system.

LBF system	Lipase activity (U/mL)	Separation efficiency (%)	Yield (%)	Purification factor
Small scale	1.83	82.0% ± 0.52	70.3	7.45
Large scale	2.56	70.1% ± 0.14	73.6	8.02

5.5 Conclusion

Microalgae *Scenedesmus* and *Desmodesmus* were used to produce lipase enzyme. The extraction and purification of lipase was performed using LBF. 400 mg microalgae biomass, 99.8% ethanol and 300 g/L ammonium sulphate combination, volume ratio of 1: 0.83, pH of 7.0, air flotation of 150 cc/min and 10 min flotation time were discovered to be optimal LBF conditions. The optimized LBF system was found to have a maximum lipase recovery of 90.4%. The recycled solvents also demonstrated satisfactory performance, indicating the possibility to reuse the solvents in the future. This could reduce the cost for the production of biomolecules from the microalgae and other microorganisms as well as minimize the environmental impact. Future research on the lipase characterization studies, such as effect of chelating agents, metal ions, substrate specificity, pH and temperature, as well as the biocatalytic and kinetic studies might extend the explanations on the mechanism of action of lipase. The current research findings will pave the way for novel discoveries and potential applications in the enzyme synthesis and purification industry.

Competing interests

The authors declare that they have no competing interests.

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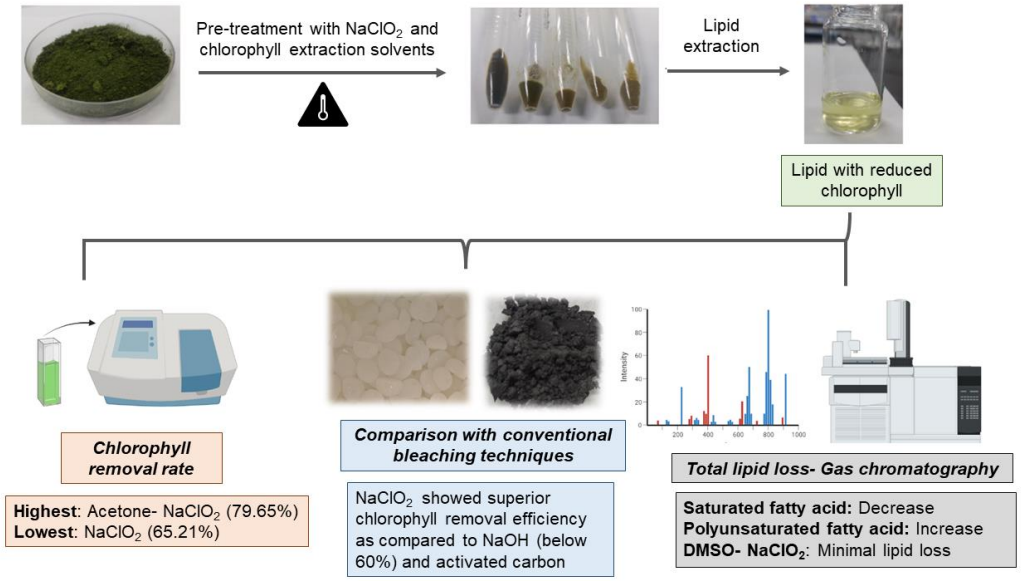
CHAPTER 6 DECHLOROPHYLLIZATION OF MICROALGAE BIOMASS FOR THE BIOCONVERSION INTO LIPID-RICH BIOPRODUCTS



This chapter focuses on the novel pre-treatment method using sodium chlorite as a pre-treatment step to remove chlorophyll from the microalgae prior lipid extraction. The lipid loss rates of various treatment methods are evaluated. This chapter consists of thesis-version of work published in the Industrial & Engineering Chemistry Research (https://doi.org/10.1021/acs.iecr.3c00780).

Dechlorophyllization of Microalgae Biomass for The Bioconversion into Lipid-Rich Bioproducts

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6.1 Abstract

Chlorophyll is one of the abundant pigments found in microalgae, which can affect the stability of its by-products. The conventional bleaching procedure involves adsorption approach, such as with clay and activated carbon, to remove chlorophyll from the oil, but this can cause disposal problems. Therefore, the present study proposed the novel pre-treatment of biomass using sodium chlorite ($NaClO_2$) to remove chlorophyll from the microalgae prior to lipid extraction. The chlorophyll reduction and lipid loss rates were evaluated. The findings revealed that approximately 70% of the chlorophyll in biomass was removed using $NaClO_2$ and chlorophyll extraction solvents. The oil yielded by chlorophyll-reduced biomass was orange-green colour and extracted oil was very fluidic. In the treated biomass, the proportion of the saturated fatty acids reduced whereas the unsaturated fatty acids level increased. Different treated biomass demonstrated varied lipid loss rate, with 13% being the lowest for DMSO-$NaClO_2$. The biochemical composition including carbohydrate and proteins in treated biomass did not affect significantly as compared to untreated biomass. In summary, the findings provide a useful pathway to remove chlorophyll that can serve as the alternative in the bleaching of microalgae oil in producing high-value lipid-based bioproducts.

Keywords: Algae biomass; Pre-treatment; Dechlorophyllization; Biorefinery; Bleaching

Synopsis:

This is the first study to investigate a pre-processing procedure that uses sodium chlorite ($NaClO_2$) to remove chlorophyll from microalgae.

Highlights

- Novel $NaClO_2$ was used to remove chlorophyll pigments from the algae biomass.
- $NaClO_2$ showed favourable chlorophyll removal efficiency, which was around 70%.
- DMSO-$NaClO_2$ retained most of the fatty acids with lipid loss rate of 13%.
- Unsaturated fatty acid content increased by 90% after treatment with $NaClO_2$.

6.2 Introduction

To extract lipid from microalgae, a number of extraction methods can be utilised, including Soxhlet extraction, Folch extraction, the Bligh and Dyer method, supercritical fluid extraction, ionic liquid extraction, and switchable green solvents [281, 299-301]. A challenging problem which arises in this domain is the presence of high pigments concentration, for example chlorophyll, in the microalgae lipid extracts. Chlorophyll has various non-polar moieties that are hydrophobic in nature, which can be extracted using organic solvent such as hexane, a common solvent of choice for lipid extractions [302]. The presence of chlorophyll in the lipid can increase the

susceptibility to photo-oxidation, decrease the storage stability, and generate an unfavourable taste [303, 304], requiring storing in dark containers. The contamination of chlorophyll will cause an overestimation in the lipids analysis test [305, 306]. As a result, isolation and removal of chlorophyll is a vital step in the commercialization of microalgae oil-based products in various industries such as biofuel, food and beverages and nutraceutical areas.

Bleaching can be accomplished through adsorption, heating, or chemical oxidation. The investigation on the removal of chlorophyll from microalgae oil is limited. Chen, Liu [307] used bleaching earth to remove chlorophyll and other impurities from crude algae biodiesel. The findings revealed that the removal efficiencies of chlorophyll and total carotene were 99.1% and 89.6%, respectively. Tang, Chew [308] compared the performance of kaolinite, Fuller's earth and bentonite clays to remove chlorophyll from the microalgae lipid feedstock and found that bentonite clay can remove chlorophyll effectively (76.04%). However, the use of bleaching clays as adsorbents to remove pigments poses several issues such as oil retention, acid waste, addition of filtration step, and environmental issues which may result in greater oil loss and clay disposal expenses. Therefore, the existing limitations of bleaching clays require the necessity of more sustainable, eco-friendly, and quick alternative chlorophyll pigment removal methods.

Pre-processing of microalgae biomass for chlorophyll removal prior extraction could become another promising line of research in removing chlorophyll from the microalgae. Chlorophyll becomes unstable when exposed to strong light, acids and bases [303]. Lipids are mostly found in within the rigid cell wall which act as the barriers to protect storage lipids, so chlorophyll removal could be carried out prior to lipid extraction to minimise lipid loss [309]. Sathish and Sims [306] has developed a wet lipid extraction procedure which was capable of removing chlorophyll from the algal lipid extract through precipitation. Li, Xu [303] also proposed a pre-processing method which can remove 96% of the chlorophyll from *Scenedesmus* sp. oil through saponification. Next, Yarnpakdee, Senphan [310] demonstrated that the preservation of bioactive chemicals in the microalgae was better achieved with the proper pre-treatments by chlorophyll removal because some phenolics, particularly non-polar molecules, may have been partially removed along with chlorophyll during the dechlorophyllization process using chloroform. Sandani, Nishshanka [309] removed chlorophyll from dry microalgae biomass using ethanol and sodium hydroxide before extracting lipids from the microalgae. Li, Xu [303] also pre-processed the microalgae biomass through saponification and showed improvements in microalgae oil quality thus lowering the cost of microalgae biodiesel. Park, Kim [311] study demonstrated that saponification of chlorophyll using high concentration of potassium

hydroxide can remove around 94 % of chlorophyll during transesterification process.

This is the first ever report to examine the efficacy of $NaClO_2$ in terms of chlorophyll reduction in the microalgae. $NaClO_2$ is a powerful oxidising and bleaching agent that has been used as food sanitation, bleaching textiles, paper pulp and etc [312, 313]. The microalgae biomass is pre-treated using $NaClO_2$ and chlorophyll extractant solvents. The organic solvents, including methanol, ethanol, acetone, dimethyl sulfoxide, are used to extract chlorophyll from microalgae because the water-insoluble chlorophyll dissolves easily in these organic solvents. Choosing the best solvent for extraction requires the consideration of the features, such as toxicity, polarity, solubility and stability. The best solvents for extracting chlorophyll depend on the microalgae species [314]. The chlorophyll removal efficiency using $NaClO_2$ is then compared with the conventional bleaching methods using activated carbon and the saponification methods using sodium hydroxide. The present study also aims to elucidate the effect of dechlorophyllization in the lipid loss rate of the extracts. The findings suggest a useful approach for removing the chlorophyll to improve the quality of microalgae oil.

6.3 Experimental setup

6.3.1 Microalgae

The microalgae used in this study was obtained from the raceway systems located in Ume, northern Sweden (63°86′ N) at Ume Energy (Ume,

Sweden) combined heat and power plant. The microalgae were cultivated using municipal untreated wastewater influent collected from the local wastewater treatment plant (Vakin, Ume, Sweden). A consortium of local freshwater green algae was collected and genetically identified as local strain of *Scenedesmus* sp. and *Desmodesmus* sp. [188].

6.3.2 Reagents

Reagents included NaOH, $NaClO_2$, methanol, ethanol, acetone and dimethyl sulfoxide were from R&M Chemical, Kumpulan Saintifik F.E. Sdn Bhd (KSFE). Fatty acid methyl esters standard mixture used in this study was obtained from Sigma-Aldrich. All reagents used were laboratory grade.

6.3.2.1 Sample preparation- Pre-treatment of microalgae biomass with $NaClO_2$

10% (w/v) of $NaClO_2$ was prepared by dissolving 10 g of $NaClO_2$ in 100 mL of distilled water. Second, 0.5 g of microalgae biomass were mixed with $NaClO_2$ and four organic solvents (acetone, methanol, DMSO and ethanol) in a beaker in 1:1 ratio (v/v) at 60 °C for 30 minutes on the hotplate. After that, the pre-treated microalgae biomass was centrifuged for 30 minutes to remove the supernatant. The residues, microalgae biomass was freeze-dried for the following experiments. The lipid extraction from treated microalgae biomass was conducted using modified Bligh and Dyer method [315], at which the lipids were extracted using dichloromethane/methanol solution (1:2, v/v). There were three

experimental replicates of each treatment (n = 3). The most effective treatment was chosen following gas chromatography analysis.

6.3.3 Biochemical Compositional and Spectrophotometric Analysis

The biochemical composition studies of carbohydrates and protein were conducted using phenol-sulphuric acid and bicinchoninic acid protein assay, respectively [316]. Using a Shimadzu UV-1800 spectrophotometer, the complete spectrum scan from 400 to 1000 nm was conducted against the blank to identify the suitable wavelength that delivered the most accurate and reliable absorbance for the measurement of chlorophyll. The absorbance at 630 nm was chosen to determine the concentration of chlorophyll. The chlorophyll removal rate was evaluated by Equation 1:

$$\text{Rate} = \frac{Initial\ absorbance\ reading - Final\ absorbance\ reading}{Initial\ absorbance\ reading} \times 100\% \quad \text{. Equation 6.1}$$

6.3.4 Lipid analysis- Gravimetric method

Approximately 50 mg of freeze-dried microalgae biomass was mixed with 3 mL of chloroform: methanol (2:1, v/v). After 30 minutes, the supernatant lipid mixture was transferred to a separate tube and heated at 60 °C for 1 hour, followed by washing step with 0.9% NaCl solution. The mixture was vortexed and resulted in the formation of two phases. The lower phase containing lipids was dried in a fume hood through the air flow at room temperature until it was fully evaporated. The total weight of lipid before and after evaporation was measured to determine the total lipid content.

6.3.5 Gas Chromatography with Flame Ionization Detection (GC-FID)

The fatty acid composition was analysed using GC-FID (Perkin Elmer Clarus 690 GC, USA). The lipids was transmethylated into fatty acid methyl ester by transmethylation with the addition of methyl dodecanoate as an internal standard. The samples were injected into an Agilent Technologies analytical laboratory instrument column (30 m x 0.25 mm x id 0.25 m) (InertCap Pure-WAX, GL Sciences). The GC-FID conditions were as follows: helium at 1.0 mL/min, injector temperature of 250 °C, detector temperature of 260 °C, and an initial column oven temperature of 35 °C with 4 °C/min increment and maintained at 220 °C for 35 minutes. The selected split ratio was 15:1, and the pressure of the carrier gas, helium was 103.4 kPa [317]. The standard involved was fatty acid methyl esters standard mixture that consisted of 13 fatty acids (methyl octanoate, methyl nonanoate, methyl decanoate, methyl laurate, methyl myristate, methyl palmitate, methyl stearate, methyl arachidate, methyl behenate, methyl tetracosanoate, methyl hexacosanoate, methyl octacosanoate, methyl triacontanoate). A standard curve was form through calibration to find the concentration of lipid of the untreated and treated microalgae biomass.

6.3.6 Statistical analysis

The statistical significance was performed using software IBM SPSS statistics 28.0.1 by applying a significance level of 0.5.

6.4 Result and discussion

Chlorophyll is a crucial and main pigment for photosynthetic microalgae. However, during the extraction process, chlorophyll is co-extracted together with the desired biomolecules [303, 318-320]. In this study, pre-treatment methods using $NaClO_2$ and organic solvents, that were ethanol, methanol, acetone and DMSO, were employed to isolate chlorophyll from microalgae biomass. The findings were then compared with the conventional bleaching techniques using activated carbon and the pre-treatment method using NaOH.

6.4.1 Colour changes of the untreated and treated microalgae biomass

The colour of the untreated and treated microalgae biomass was shown in **Figure 6.1**. Based on **Figure 6.1**, the colour of microalgae biomass treated only with $NaClO_2$ showed a green-orange colour. The microalgae biomass was dark orange colour after being treated with $NaClO_2$ and chlorophyll extractant organic solvents. It was evident that the efficacy of ethanol, acetone, methanol, and DMSO to extract chlorophyll from the microalgae varied. The result of this colour analysis was then compared with the study by Li, Xu [303] which used saponification method to pre-treat the microalgae biomass to remove chlorophyll. The result demonstrated that the chlorophyll-reduced biomass showed a yellow-green, orange colour, indicating the chlorophyll had been removed from the biomass. In short,

comparable to NaOH, $NaClO_2$ was also effective to remove chlorophyll from the microalgae biomass.

6.4.2 Chlorophyll removal efficiency

The chlorophyll removal rate was experimentally investigated. From **Table 6.1**, the chlorophyll removal rate ranged from 53 % to 79 %. Acetone-$NaClO_2$ demonstrated the highest chlorophyll removal rate with 79.65% whereas treatment with $NaClO_2$ showed the lowest chlorophyll removal efficiency (65.21%). The chlorophyll removal rate was then compared with the saponification method involving sodium hydroxide to remove chlorophyll. The finding revealed that the chlorophyll removal rate of treatment with sodium hydroxide and chlorophyll extractant solvents were all below 60%. A similar pattern of results was obtained in Baroi and Dalai [321] study that employed activated clay to remove chlorophyll from green seed canola oil at a rate of 75.56%. The study by Sandani, Nishshanka [309] demonstrated chlorophyll removal efficiency of 67.5 % through pre-treatment process of *Chlorella homosphaera* using ethanol and NaOH. From the results, $NaClO_2$ was capable of removing chlorophyll from the microalgae biomass. Future studies could explore this issue further by increasing the concentration of $NaClO_2$ or the incorporation of ultrasound technique to increase the removal rate above 90%. Numerous prior investigations had demonstrated that organic solvents may remove the pigments with an efficiency of more than 95% [322] but the effectiveness may varied depending to the microalgae species and extraction

circumstances [323]. The results demonstrated that pre-treatment of microalgae biomass, in this study using $NaClO_2$, prior to lipid extraction aided in chlorophyll removal.

6.4.3 Comparison of chlorophyll removal efficiency with conventional bleaching method

The low-cost adsorbents, such as activated carbon, silica gel and bleaching clay, were used to adsorb chlorophyll and other pigments from the oil extract [314, 324]. The chlorophyll removal rate of treated microalgae biomass was compared to the traditional bleaching method using activated carbon which was applied after the lipid extraction. Nevertheless, despite the satisfactory effect of activated carbon on chlorophyll removal, this study found that it also resulted in a lipid loss rate of about 80%. This is because chlorophyll was covalently coupled with biomolecules, such as lipid and protein, and separated or eluted together by physical absorption. The study by Phaisan, Yusakul [325] claimed that the use of activated carbon in the natural product research and development had the disadvantage of causing non-specific adsorption, which results in the loss of desired bioactive compounds. Moreover, chlorophyll removal using adsorbents was time-consuming and generates a lot of solid waste [324, 326].

6.4.4 Biochemical composition of treated biomass

The biochemical composition analysis of the treated microalgae biomass, such as lipids, carbohydrates, and proteins, was analysed. The total

carbohydrates and proteins in treated biomass with $NaClO_2$ were decreased to varied degree by 20-30% ($p > 0.05$) as compared to untreated biomass. The findings were directly in line with previous finding by Li, Xu [303] which showed that the pre-processing of *Scenedesmus* sp. with ethanol-NaOH reduced approximately 25% of biomass weight, 23% total lipid, 33% of carbohydrates, 43% of proteins and 2% of other compounds such as RNA and DNA.

6.4.5 Lipid retention

There was another concern that the organic solvents would extract the lipids along with the chlorophyll during the bleaching process, so it was essential to examine the total lipid loss rate. To extract lipids from the organisms, the solvents, such as methanol, ethanol, chloroform, hexane, and acetone, are extensively used in Folch, Bligh and Dyer and other lipid extraction techniques. These solvents may result in significant lipid loss because they can dissolve liposoluble compounds. Sandani, Nishshanka [309] had demonstrated that the lipid loss rate of 44.2% when compared to the control samples without chlorophyll removal. Nevertheless, these solvents had the greatest potential for extracting chlorophyll. It is critical to study the lipid loss rate for the maximum lipid recovery as well as the selection of the solvents with high affinity to the chlorophyll but less favoured towards lipids throughout bleaching process [327, 328].

The microalgae oil recovered from the $NaClO_2$-treated microalgae were bright orange-green. The FAME profile of lipid extracts will be

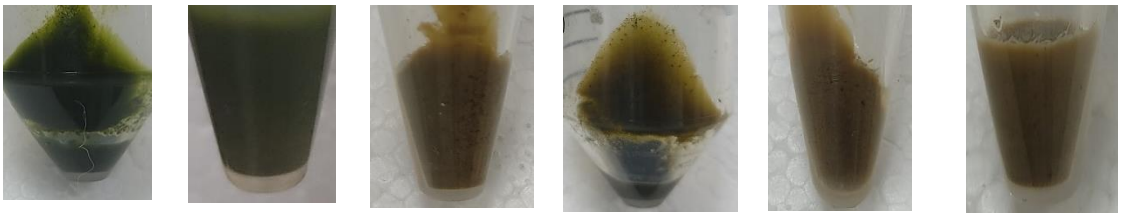
changed due to the structural modifications to the cell wall and lipid globules caused by pre-treatment process [309]. Hence, each lipid fractionation of the treated and untreated microalgae biomass was examined using GC-FID. Through standard curve calibration, GC-FID can confirm the identity and quantity of fatty acids based on carbon number. The calibration curve from lipid standard was calibrated with the R^2 of 0.9891. The fatty acid composition of untreated oil was represented in **Table 6.1**. The findings revealed that untreated oil primarily consisted of saturated fatty acid (lauric acid, palmitic acid, stearic acid, behenic acid) and unsaturated fatty acid (arachidonic acid, polyunsaturated omega-6 fatty acid 20:4). The total lipid loss rate was: DMSO (13.42 %) < ethanol (33.39%) < $NaClO_2$ (39.14%) < acetone (59.21%) < methanol (73.40%).

In the untreated biomass, there were five methyl esters detected, which were methyl laurate, methyl palmitate, methyl stearate, methyl arachidate and methyl behenate. However, methyl laurate, methyl palmitate and methyl behenate were not detected in the treated biomass. Among the five treatment methods, saturated fatty acid composition decreased significantly while at the same time, polyunsaturated fatty acid increased significantly. DMSO-$NaClO_2$ treatment showed the satisfactory performance with the minimum lipid loss. In DMSO-$NaClO_2$ treatment, the proportion of saturated fatty acids was decreased by 89.68% whereas polyunsaturated fatty acids increased greatly by 92.5% ($p < 0.05$). According to our findings, the total lipid of treated biomass decreased

because of the use of combination of $NaClO_2$ and organic solvents during the chlorophyll removal. The study by Li, Xu [303] demonstrated that the ethanol-NaOH pre-treatment of microalgae biomass in the chlorophyll removal experiment resulted in a less than 3% decrease in the proportion of saturated fatty acids and unsaturated fatty acids in neutral lipids. Sandani, Nishshanka [309] study also showed a significant reduction in polyunsaturated fatty acid concentration in pre-treated *Chlorella homosphaera* biomass using ethanol and NaOH. The current study showed that the reduction in chlorophyll was greater than the reduction in total lipid, with the exception of $NaClO_2$-methanol, which was greater than 70%. Superior results were obtained for $NaClO_2$-DMSO and $NaClO_2$-ethanol which result in less than 30% of lipid loss because the considerable decrease in total lipid in the treated biomass could result in significant economic losses if employed on a big scale.

Overall, the findings of this study suggested that the combination of DMSO and $NaClO_2$ could become the suitable and most effective approach for removing chlorophyll. Future research should consider the optimisation process to reduce total lipid loss below 10%, for example, temperature, duration, concentration, and volume ratio. This is because altering and optimising the process conditions through an increase in the concentration of reactant, time and temperature of the system can enhance the rate of chemical reaction, boost up the performance of chlorophyll removal from the microalgae biomass, producing better results,

cost reduction while sustaining the lipid compounds. Finding a balance between lipid loss and chlorophyll removal by optimising bleaching conditions is considered as an optimal bleaching concept. However, a high concentration of $NaClO_2$ is extremely corrosive to the equipment. Thus, it is advisable to use ceramic, plastic, or special steel for bleaching process [329]. Future studies could also investigate the cytological mechanism of chlorophyll removal by comparing the morphological and ultrastructural alterations in treated and untreated microalgae cells [303]. The suggested strategy would significantly advance the technology for fully and completely utilising microalgal biomass to produce oil feedstocks as biofuel and other bioproducts, such as pharmaceuticals drugs, food additives and health supplements.



(a) **(b)** **(c)** **(d)** **(e)** **(f)**

Figure 6.1. Colour changes of microalgae biomass after the treatment with $NaClO_2$ and various chlorophyll extractant solvents: (a) Control; (b) $NaClO_2$; (c) Methanol + $NaClO_2$; (d) DMSO + $NaClO_2$; (e) Ethanol + $NaClO_2$; (f) Acetone + $NaClO_2$

Table 6.1. Chlorophyll removal using various pre-treatment techniques from the microalgae biomass.

Pre-treatment step	Chlorophyll removal rate (%)	Gravimetric analysis	Fatty acid composition (% TFA)				
			Lauric acid	Palmitic acid	Octadecanoic (stearic) acid	Arachidic acid	Behenic acid
a) Control	-	8.57	0.15	0.23	73.78	25.39	0.45
b) $NaClO_2$	65.21	6.70	NA	1.38	30.50	68.12	NA
c) Methanol + $NaClO_2$	75.74	7.03	NA	NA	42.23	57.77	NA
d) DMSO + $NaClO_2$	68.98	7.42	NA	NA	3.45	96.55	NA

e) Ethanol + $NaClO_2$	71.68	7.32	NA	1.03	22.87	76.10	NA
f) Acetone + $NaClO_2$	79.65	7.98	NA	10.63	29.70	59.67	NA

*** NA = The lipid is not detected in the microalgae biomass.**

6.5 Conclusion

There is a growing trend to investigate the approaches for removing chlorophyll from the microalgae oils due to its negative consequences on downstream processing and oil quality in the production of oil and other valuable products from microalgal biomass. A pre-treatment method using $NaClO_2$ to remove chlorophyll from the microalgae was investigated. The microalgae oil refining ability of the pre-treatment method using $NaClO_2$ was compared with the conventional bleaching technique using activated carbon. The current investigation revealed that $NaClO_2$ and organic chlorophyll extractant solvents had a satisfactory ability for extracting chlorophyll. DMSO-$NaClO_2$ is chosen as the most effective way for removing chlorophyll and its derived components from the microalgae biomass and minimising the amount of total lipids lost during the bleaching process. However, the choice of suitable chlorophyll extractant solvents and retaining the lipid in the microalgae biomass is dependent on the microalgae species and extraction conditions. The outcome showed the potential of $NaClO_2$ pre-treatment approach in enhancing the production of microalgae oil. This will also contribute significantly to the comprehensive utilisation of the lipid produced from microalgae in cosmetics, dietary supplements, as well as biofuel production. The removal of chlorophyll allows for the manufacture of stable and yellow-coloured oil that is palatable to consumers. Future research should focus on the side effects, reusability of $NaClO_2$, reaction mechanism involved, process optimisation,

and the search for more effective approach to further enhance the removal of chlorophyll in the process of microalgae oil technology.

Author contribution statement

Doris Ying Ying Tang: Conceptualization; Methodology; Data curation; Formal analysis; Writing – original draft. **Kit Wayne Chew**: Validation; Methodology; Project administration; Writing – review & editing. **Chongqing Wang, Heli Siti Halimatul Munawaroh, Zengling Ma, Fubao Sun, Muthusamy Govarthanan, Sarah Alharthi**: Visualization; Writing – review & editing. **Pau Loke Show**: Project administration; Resources; Writing – review & editing.

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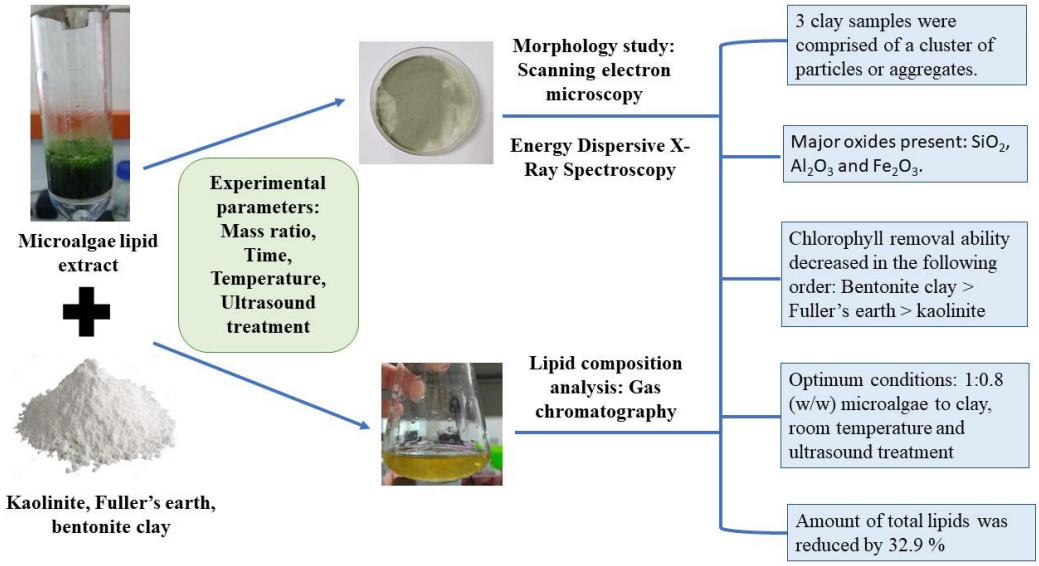
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

CHAPTER 7 PERFORMANCE OF BLEACHING CLAYS IN DECHLOROPHYLLISATION OF MICROALGAL OIL: A COMPARATIVE STUDY



This chapter compares the effectiveness of various clay types (kaolinite, Fuller's earth and bentonite clay) in removing chlorophyll from the microalgae oil. which are activated by acid treatment. Various process conditions such as the temperature, contact time and ultrasound treatment are evaluated for maximum chlorophyll removal efficiency. The reusability of the bleaching clay is also discussed in this chapter. This chapter consists of thesis-version of work published in the Process Biochemistry (https://doi.org/10.1016/j.procbio.2023.03.002).

Performance of Bleaching Clays in Dechlorophyllisation of Microalgal Oil: A Comparative Study

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7.1 Abstract

While clay has been extensively studied for its ability to remove chlorophyll from vegetable oil, its effects and suitability on microalgae oil have received far less attention. In this study, three different clay types are evaluated and compared for their ability to dechlorophyllise microalgae oil. The clays involved are kaolinite, Fuller's earth and bentonite clay which are activated by acid treatment. Bentonite clay is found to remove chlorophyll effectively (76.04%) than kaolinite and Fuller's earth due to the presence of porous materials that facilitate adsorption. Additionally, the conditions of high temperature (79.69%) and ultrasound treatment (84.24%) demonstrate satisfactory chlorophyll removal efficiency. Another promising finding is that chlorophyll can be adsorbed on the clays without affecting the lipid composition in the microalgae extract significantly. Spent bleaching clay disposal is a growing issue that poses an environmental burden. Reusing bleaching clay can lower expenses and increase oil recovery. In a single sequential run, using spent bentonite clay yields the outcomes comparable to those of virgin bentonite clay. This finding offers a useful green and environmentally friendly approach to remove chlorophyll from microalgae oil feedstock.

Practical applications

Microalgae extracts can be used in food and beverage as well as pharmaceutical sectors by replacing the artificial additives. However, there

is a significant issue that affects the microalgae-based products, particularly the use of microalgal oil in foods and supplements, which is the oxidation reaction that may result in deterioration and safety concern. Next, the use of microalgae as food and supplements is limited due to their appearance characteristic of green colour. The appearance characteristics of food or supplement products is the crucial aspect that can influence both consumer acceptability and purchasing intent. Hence, dechlorophyllisation of microalgae extract prior to extraction and purification can help to solve these issues. To the best of our knowledge, this is the first study to examine how clays can be used to remove chlorophyll from the microalgae lipid extract with non-significant losses on the fatty acids. The findings can cast a new light on the preparation of the microalgae extracts without discoloration problem in a green and environmentally approach.

Keywords: Bentonite; Adsorption; Decolourization; Spent bleaching clay; Regeneration

List of abbreviation

EDX	Energy-dispersive X-ray spectroscopy
FESEM	Field emission scanning electron microscope
SEM	Scanning electron microscopy

UV-Vis	ultraviolet-visible
XRD	X-ray powder diffraction

7.2 Introduction

Chlorophyll is made up of porphyrin rings made up carbon, hydrogen, oxygen and magnesium atoms. The process of chlorophyll removal from edible oil is important as the chlorophyll act as photo-prooxidants, reducing the oxidative stability aside from producing unappealing and unattractive dark greenish colour [330, 331]. However, the existence of a considerable amount of chlorophyll is one of the challenges faced during the lipid extraction because chlorophyll is a photosensitive pigment that can be photo-oxidized and works as a prooxidant in oils. Besides, the oil derived from microalgae have a greenish colour appearance which can reduce its appeal for product development and undesirable for consumers and thus affecting the purchasing power. Hence, chlorophyll removal should be considered as a crucial step in the use of microalgae oil for commercial purpose.

Numerous studies have been conducted to investigate how to remove chlorophyll from various oil feedstocks, for example physical adsorption, oxidation treatment, chromatography as well as the use of chemicals for example, mineral acids, hexane, chloroform and carbon tetrachloride. Baroi and Dalai [321] used the solid acid catalysts to remove

approximately 75.56 % of chlorophyll from green seed canola oil. Next, to remove chlorophyll from canola oil, Bahmaei, sadat Sabbaghian [332] precipitated the chlorophyll components using mineral acids. By combining crude canola oil with mixture of phosphoric acid and sulphuric acid, it was possible to reduce the concentration of chlorophyll from 30 ppm to less than 0.01 ppm. Kim, Bisson [333] then reported a quick efficient method named as Centrifugal Partition Chromatography (CPC) utilising a solvent system made up of hexanes–EtOAc–MeOH–water to remove chlorophyll from plant materials. Besides, Phaisan, Yusakul [325] developed an oil-based system, such as palm oil, coconut oil, rice bran oil, soybean oil, corn oil, sunflower oil and canola oil, to partition chlorophyll from the extract of *Chromolaena odorata* to the oil phase without affecting flavonoids and phenolic contents and safer than the conventional partitioning using hexane. The efficacy of chlorophyll removal can reach more than 85%. Sathish and Sims [306] used wet lipid extraction procedure to remove chlorophyll contamination effectively from the algal lipid extract through precipitation. Lastly, Li [334] pre-treated *Scenedesmus* sp. algae oil feedstock to remove 96% of chlorophyll in biomass by saponification. A closer look to the previous literature studies on chlorophyll removal reveals several gaps and shortcomings on these approaches such as the use of toxic and hazardous chemicals, expensive and time-consuming.

There are many existing adsorbent materials such as activated carbon, activated clay, silica gels, alumina and biochar. Among these,

activated clay has been used to refine the oil feedstock [331, 335-338] due to its availability and low cost as compared to other materials. Hence, in this study, natural bleaching clay powders are proposed to remove chlorophyll from microalgae oil as adsorbent materials. These clay powders selected are obtained from natural sources which show the potential to adsorb impurities from oil grease. In comparison to chlorophyll separation using organic solvents, the use of clay powders does not require the use of hazardous organic solvents and is environmentally safe [339, 340]. Past literature studies show that clay is effective in removing chlorophyll from various oils, including vegetable oils. Natural clay must be activated with an acid or alkali solution to improve its adsorption ability as the activated clay demonstrates satisfactory bleaching performance than natural clay [335-337, 341]. Salawudeen, Arinkoola [337] proved that clay activation will eliminate some interlayer cations, creating new active sites on the clay surface and thus enhancing the bleaching properties. According to the literature studies, the adsorption of chlorophyll on acid-activated clay was improved due to the chemical interaction of chlorophyll with the Lewis and Bronsted acid sites of clay [336]. Clay activation by acid may also increase the porosity, surface area and intergranular spacing, which allowed for advantageous hydrogen bonding between the heteroatoms of the pigments found for example, oxygen and nitrogen in the oil with the hydrogen atoms in acid-activated clays [335, 342, 343], enhancing the removal capability. As an example, Güler and Tunç [336]

used acid-activated clay to adsorb chlorophyll from hexane solution comprised of spinach leaves. Chen, Liu [307] employed bleaching earth to remove the impurities, chlorophyll and carotene, from the microalgae extract in the production of biodiesel. The concentration of chlorophyll dropped from 4296.7 ppm to 40.3 ppm with removal efficiency of 99.1%. This study had exclusively focused on the use of bleaching earth in removing chlorophyll. The removal of pigments, especially chlorophyll, and other contaminants by the adsorption process is one of the most significant steps in the oil refining. This process makes the oil more pleasing and suitable for consumption.

To the best of the author's knowledge, the effects of bleaching clays in dechlorophyllisation studies from the microalgae lipid extracts are rarely analysed and investigated. The current study aims to investigate the abilities of various types of bleaching clays in removing chlorophyll from the microalgae extracts. The bleaching clays involved are bentonite clay, kaolinite, and Fuller's Earth. The process of chlorophyll removal from the microalgae is optimized using the parameters which are ratio of microalgae extracts and clay powder, temperature, contact time, and the combination of ultrasonic treatment. Ultrasonication can cause dramatic changes in the structure of the minerals studied, but it also have a significant impact on their textural characteristics [344]. The adsorption effect that results in a decrease in absorbance at 670 nm is used as a measure of the amount of chlorophyll being absorbed. It is hypothesized that chlorophyll can be

adsorbed on these clay powders more efficiently without affecting the lipid composition in the microalgae extract. Finally, the spent bleaching clay is studied for the reusability for future experiment to prevent waste and save cost.

7.3 Methodology

7.3.1 Materials

Freeze dried *Desmodesmus* sp. and *Scenedesmus* sp. microalgae powder was a generous gift of Dr. Francesco Gentili (Swedish University of Agricultural Sciences). The microalgae was collected from Umeå, northern Sweden (63°86′ N) at Umeå Energy combined heat and power plant (CHP-plant) at which the microalgae was grown in municipal untreated wastewater influent [188]. Three types of clay powders utilised in this study as adsorbent materials were bentonite clay, kaolinite/ kaolin clay/ China clay and Fuller's Earth /Multani Mitti. All the consumables (beaker, test tubes, pipette tips, filter paper, cuvette) and chemical reagents (dichloromethane, methanol) were of analytical grade.

7.3.2 Methods

7.3.2.1 Removal of chlorophyll in microalgae extracts

Microalgal lipid was extracted through Bligh and Dyer method using dichloromethane and methanol as the solvents. The bleaching procedure was started with the mixing of microalgae lipid extracts using hotplate magnetic stirrer (Thermo Scientific™ Cimarec+™ Stirring Hotplates

Series) with the clay in the ratio of 1:1 (wt.%) at room temperature in 15 minutes. After that, the mixture was separated using vacuum filtration. The filter, clay powder was left on the top of the funnel to dry. The absorbance of microalgae lipid extracts before and after the treatment was measured at 660 nm using ultraviolet-visible (UV-Vis) spectrophotometer (Shimadzu UV-1800 UV). The chlorophyll removal efficiency was evaluated by Equation 1:

$$\text{Efficiency} = \frac{Initial\ absorbance\ reading - Final\ absorbance\ reading}{Initial\ absorbance\ reading} \times 100\% \quad (7.1)$$

7.3.2.2 Effect of experimental parameters

The experimental conditions such as ratio of microalgae extract to clay powder, contact time, temperature and ultrasound treatment were evaluated to obtain the optimized parameters and maximum chlorophyll removal efficiency.

7.3.2.2.1 Ratio of microalgae extracts to clay powder

The ratio of microalgae lipid extract to clay powder was set as 1:0.2, 1:0.4, 1:0.6, 1:0.8, and 1:1 to assess the optimum mass of clay powder that needed for maximum removal of chlorophyll.

7.3.2.2.2 Heating temperature

The mixture of microalgae and clay were heated to 50 °C, 100 °C and 150 °C by adjusting the temperature at the temperature control knob of hotplate magnetic stirrer.

7.3.2.2.3 Contact time

The optimal contact time was determined by conducting experiments at room temperature for 0.25, 0.5, and 1 hour of heating.

7.3.2.2.4 Ultrasound treatment

Ultrasound treatment was conducted using Bandelin Sonopuls HD 2200 Homogeniser (Germany). An ultrasonication probe was inserted into the mixture inside a beaker at room temperature. The ultrasonic probe with titanium flat tip TT 13 was operated at constant amplitude with pulse mode of 10 s ON/ 30 s OFF for total duration of 10 minutes [345].

7.3.2.3 Analysis and Characterization Study

Scanning electron microscope (Model: FEI Quanta 400F) coupled with energy dispersive X-ray spectroscopy (Model: Oxford- Instruments INCA 400 with X-Max Detector) were used to determine the morphology and elements of clay powders. To examine the physical structure of clays, the clay samples after filtration were left dried at room temperature. Moreover, the lipid composition in the microalgae extracts was determined using the gas chromatography with flame ionization detection (Clarus 690 GC, Perkin Elmer). The lipid standard used in this study was F.A.M.E. Mix, C4-C24, certified reference material (Sigma Aldrich). To quantify the lipid composition before and after treatment, a calibration curve was plotted. The R^2 of calibration was 0.9891.

7.3.3 Statistical analysis

The experiments were repeated three times to increase the accuracy and precision of the result. The result was expressed as means from triplicate experiments ± standard error. The significance test was analysed using analysis of variance (ANOVA) and significance level at p value less than 0.05 using software IBM SPSS Statistics. The graph with error bars was plotted using Sigma Plot version 12.0.

7.4 Result and discussion

7.4.1 Characterisation of adsorbent

7.4.1.1 Scanning electron microscopy (SEM)

In this study, three bleaching clay samples, bentonite clay, kaolinite and Fuller's earth were selected to investigate their ability to remove chlorophyll from microalgae lipid extracts. Prior to the study, the three study bleaching clays were activated with mineral acid to improve chlorophyll removal efficiency.

To further understand the decolorization capabilities, the porosity and pore size distribution in kaolinite, bentonite and Fuller's earth samples were studied. SEM micrographs of three study clays were illustrated as **Figure 7.1**. This SEM analysis found evidence for that the three clay samples were comprised of a cluster of particles or aggregates. According to the micrographs, bentonite (**Figure 7.1a**) and Fuller's earth (**Figure 7.1c**) exhibited distinct porous texture. Next, SEM examination of kaolinite (**Figure 7.1b**) revealed irregular and stacked or mats of flakes in the form

of agglomerates. A similar pattern of results was obtained in the studies by Ullah, Hussain [346] and Worasith, Goodman [347] that SEM of bentonite and kaolinite displayed agglomerated and platy characteristics, respectively.

The adsorption ability of the clay towards certain compound depends on the accessibility of the molecules into the pores, surface area, pores size and clay mineral types. There are three different types of pores that distinguish based on pore size or diameter, which are micropores, mesopores and macropores. Micropores are defined as having a width of less than 2 nm; mesopores are the pore with the width of between 2 and 50 nm, and lastly, macropores are defined as having a width greater than 50 nm. By comparing adsorption capability, the performance of macropores is greater as compared to micropores and mesopores due to high surface area and increasing pore volume [348]. From **Figure 7.1**, activated bentonite and Fuller's earth comprised of a lot macrospores [348] which believed contributed to their high adsorption capability. Kaolin particles are primarily in the 25–35 μm size range, whereas only a small number of particles have a size distribution that varies between 0.4-0.75 μm [347, 349-351]. Moreover, the size of the adsorbed material can affect the adsorption capacity as the large particle size was difficult to be embedded or fit into the small pore size of adsorbent. One concern about the findings was that it was challenging to pinpoint the specific texture and morphology of the clays through SEM due to particle coalescence. In

short, the surface area and porosity or pore size of the bleaching clay can influence the amount of chlorophyll absorbed.

7.4.1.2 X-ray powder diffraction (XRD)

The major, minor and trace compounds in three studied clays were identified. The mineralogical composition of activated clay samples was presented in **Figure 7.2** and **Table 7.1**. The result revealed that the major oxides present in the three clay samples were SiO_2, Al_2O_3 and Fe_2O_3. Calcium oxide, potassium oxide and sodium oxide were present in trace amounts. This was supported by the previous study by Rezapour, Abdollahi [352] that the activated bentonite consisted mainly of aluminium oxide and silicon dioxide. Sodium oxide, magnesium oxide, potassium oxide and iron (III) oxide were the minor oxides. Next, Panda, Mishra [350] conducted X-ray fluorescence to determine the chemical composition of kaolin clay and found out that alumina and silica made up the majority of kaolin clay with minor amount of magnesium oxide, calcium oxide, zinc oxide and potassium oxide. Worasith, Goodman [347] study also produced similar result, at which Si, Al, O were the major elements in kaolin sample.

7.4.2 Adsorption Kinetics Experiment

Fuller's earth, or also known as bleaching earth, is the most popular and utilised mostly in the vegetable oil bleaching process since the end of the 18th century [353]. Bentonite clay and Fuller's earth are considered as good adsorbent and the compositions of both clays are almost similar, except different forms of silicates. Additionally, bentonite, which is created

from old volcanic ashes, can be found in Fuller's earth. Kaolin clay also recently used for oil decolourisation as adsorbent of impurities [347, 349, 350]. In the present study, the removal ability of chlorophyll from the microalgae extracts decreased in the following order: Bentonite clay > Fuller's earth > kaolinite (**Table 7.2**). When comparing the result to the literature study by Usman, Oribayo [351], it was pointed out that the bleaching rate of activated bentonite was higher (81.4%) than that of kaolin clay (62.4%). The adsorption capacity of kaolin clay was low as compared to other clay materials due to low surface charge and small surface area (8-15m^2/g) [353]. The adsorption capabilities of the clay, especially bentonite and Fuller's earth, was presentable because the majority of these bleaching earths are aluminium silicates, which are typically montmorillonite clays with adsorptive qualities in both their inactivated and activated states [351]. Hence, bentonite clay was chosen for further optimization study because of the satisfactory chlorophyll capacity. A few parameters were explored and optimized to obtain the highest chlorophyll removal efficiency. The variables examined were the ratio of microalgae extracts, contact time, temperature and combination of ultrasound treatment as shown in **Figure 7.3**. The reduction in colour and oil retention by the bleaching clay was used to monitor bleaching efficiency. Pigments, primarily chlorophylls, make up the colour bodies of microalgae oils. The reduction of colour bodies in this work was measured using UV-vis method. In conclusion, bentonite clay produced satisfactory chlorophyll

removal and was employed for the subsequent adsorption kinetics analysis.

7.4.2.1 Effect of clay load

The relationship between the concentration of clay and microalgae biomass with the removal rate of chlorophyll are important to be assessed. A change in the clay and microalgae feedstock concentration will affect how well the chlorophyll removal performance work. Therefore, in this experiment, the influence of clay concentration ranging from 20 wt.% to 100 wt.% was examined. **Figure 7.3a** demonstrated that clay load with 1:1 ratio produced the highest chlorophyll removal efficiency, which was 76.04%. For the clay load, the removal efficiency increased dramatically from ratio of 1:0.2 to 1: 0.8 and increased slightly beyond ratio of 1:0.8. It can be assumed that the amount of chlorophyll removed was increased because large adsorbent amount provided more adsorption sites, increasing the adsorption of chlorophyll molecules. Beyond the ratio of 1:0.8, the removal rate was increased slightly and then remained stable because the clay was saturated with the chlorophyll. Because the adsorption equilibrium between the adsorbent and the oil had been reached, additional pigment removal by the excess adsorbent dosage was not possible. Usman, Oribayo [351] came to a similar conclusion when using Afashio kaolin and local bentonite in palm oil bleaching. El-Hamidi and Zaher [331] study also showed that the removal efficiency of low clay load (1 wt.%) of Mexican clay and Tonsil ACC with high temperature was

significant. Therefore, the optimized clay load for the removal efficiency of chlorophyll from the microalgae was selected to be 1:0.8 ratio of microalgae lipid extracts to clay samples.

7.4.2.2 Effect of temperature

Similarly, temperature is important in chlorophyll removal as the changes in temperature will cause the alteration in adsorption of chlorophyll in the clay. The adsorption kinetics and oil viscosity are influenced by temperature. Oil becomes less viscous as temperature rises, causing dispersion of clay particles and better interaction of clay and oil. Thus, flow resistance is reduced [354].

In this study, the microalgae extracts and clay heated to 50 °C, 100 °C and 150 °C. **Figure 7.3b** illustrated the effect of temperature on the chlorophyll removal efficiency. The results showed that the chlorophyll removal rate was minimally different, with the heating temperature of 150 °C demonstrating the highest chlorophyll removal efficiency, which was 79.69%. Together, the present finding confirmed that longer heating duration will also increase the chlorophyll removal efficiency. **Figure 7.3d** demonstrated the effect of high temperature on the morphology of bentonite clay. High temperature caused structural changes at which the pore apertures increased and large increase of pore volume, proposing high adsorption ability. Despite that the highest temperature showed the satisfactory removal efficiency, but the obstacles faced during the process was loss of the solvents during heating, result in the less amount of final

product. The volume of final products reduced by 70%. In microalgae, lipid extraction was performed using methanol and dichloromethane. The boiling point of methanol is 64.7 °C whereas the boiling point of dichloromethane is 39.6 °C. Due to high volatility, methanol and dichloromethane are expected to evaporate at high temperature easily and rapidly. This result tied well with previous study [331] wherein the high temperature can enhance the absorption efficiency at lower clay load but was dependent on clay type. However, the chlorophyll removal efficiency will not significantly improve as the bleaching temperature. Hence, to reduce energy consumption and the subsequent operating costs, it was not advised to heat oils during the bleaching process [331]. Besides, high heating temperature may damage the oxidative stability of the edible oils seriously [335]. Hence, room temperature was chosen as the optimized temperature to remove chlorophyll efficiently.

7.4.2.3 Effect of contact time

In addition, contact time is crucial for the removal of chlorophyll. The efficiency of chlorophyll removal will increase as the contact time rises until a stationary phase is reached due to the limited capacity. Once it reached threshold point, increase in contact time would not improve the efficiency of removal. In other words, as the medium approaches equilibrium, the bleaching efficiency began to stabilise or become steady as the contact time increased. This study looked into the impact of contact time ranging from 15 minutes to 60 minutes in room temperature to find a feasible

equilibrium contact time. **Figure 7.3** showed that the longer contact time, the higher chlorophyl removal ability. The highest chlorophyll removal activity was found to be 80.72% with 1 hour of contact time. After 30 minutes, the chlorophyll was removed at a slower rate due to saturation of available sites and remained nearly constant after 60 minutes. Nevertheless, long contact times require considerable energy usage, which is expensive, unfriendly to the environment, and unsuitable for large-scale procedures. Hence, from this study, the optimal contact time was found to be 30 minutes.

7.4.2.4 Effect of ultrasound treatment

The impact of ultrasound-assisted technology along with the use of clay in the chlorophyll removal ability was investigated. The benefit of using ultrasound treatment is to reduce the duration of the entire bleaching process. The results of the experiment found clear support for the ultrasound treatment (84.24% ± 1.251) in high chlorophyll removal efficiency as compared to that without ultrasound treatment (76.04% ± 0.0212). FESEM study (**Figure 7.1e**) further supported this finding. Ultrasound strongly altered the textural morphology of the bentonite clay. The enormous shear forces generated by the cavitation bubbles of ultrasound waves destroyed the clay structure by enlarge the pore volume and diameter [355], improving the chlorophyll removing capabilities from the microalgae extracts. The study by Liang, Aachary [338] showed that the combination of ultrasonic treatment of cold pressed hempseed oil with

bleaching clays was rapid and reduced chlorophyll content significantly. Aachary, Liang [356] also showed that after ultrasonic bleaching with clays, hempseed chlorophyll was significantly reduced as well as changed hempseed oil's colour during the ultrasonic bleaching. The influence of ultrasound exposure of clays with regards to the exposure time, duty cycle, intensity and frequency in the chlorophyll removal rate can be investigated in the future.

7.4.3 Changes in lipid composition

From the aforementioned data, the lipid composition of filtrate at which the chlorophyll was removed using the optimized conditions from the microalgae extract was then examined. Since clay is an absorbent, it is of interest to know whether the clay will adsorb lipid or fatty acids in addition to chlorophyll and other impurities. The fatty acids in the samples were analysed using gas chromatography to help with this topic. The lipid composition before and after the addition of clay was compared, and it was found out that the amount of total lipids was reduced by 32.9 %. Literature studies showed that the analysis of spent bleaching earth comprised of 20–40 wt.% of residual vegetable oil, pigments, oxidation by-products, free fatty acids (FFA), impurities and contaminants [357, 358], suggested that lipid amount will be reduced during the decolourization process. Moreover, this finding was in accordance with finding reported by Su, Wang [359] that palmitic acid, oleic acid and linoleic acid decreased by about 37%. It had been shown that free fatty acids can be adsorbed onto acid-activated clay.

However, since the fatty acids were only partially eliminated, it appeared that clay removed the chlorophyll much more selectively than fatty acids.

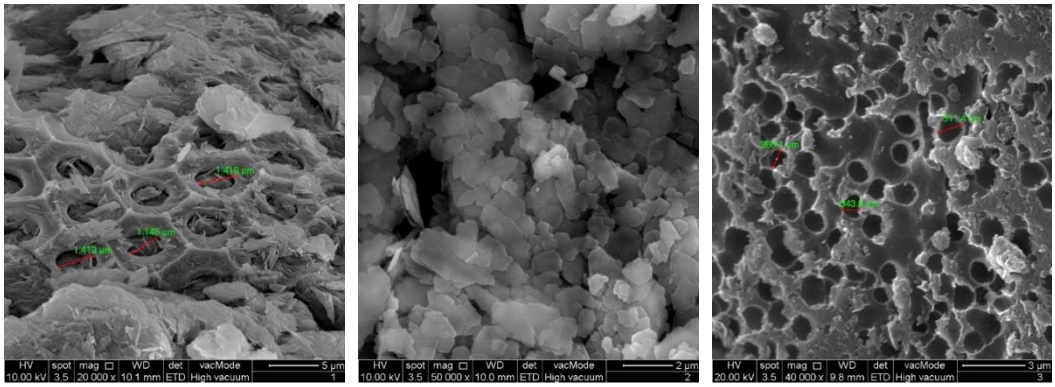
7.4.4 Reusability of clay

The edible oil sector generates more than 2 million tonnes of spent bleaching earth per year globally, with the majority produced in middle east countries which produce significant amounts of edible oil [360]. Hence, in this study, the reusability of spent bleaching clay also being evaluated to save the cost. The spent bleaching clay was soaked in acetone and hexane to remove chlorophyll and lipid fraction, respectively, followed by drying process in the oven. The spent clay was then reactivated using mineral acid and assessed the removal ability of chlorophyll using the optimized conditions. **Table 7.2** demonstrates the performances of reactivated spent bleaching clay in one sequential run for chlorophyll removal efficiency. The findings revealed that chlorophyll removal efficiency of reactivated spent bentonite clay was overwhelming as compared to other used bleaching clays and produced similar result as the virgin activated bentonite. This showed that after the bleaching process, the spent clay can be reused to minimize the waste and save cost. Besides, the solvents involved in the reactivation of clay also can be reused, for example acetone and hexane through rotary evaporation to remove the residual. There are negligible or minimal losses of lipids that occurred during the recycling steps, thereby demonstrating the benefits of utilizing recycled clay for chlorophyll removal in the industries. Su, Wang

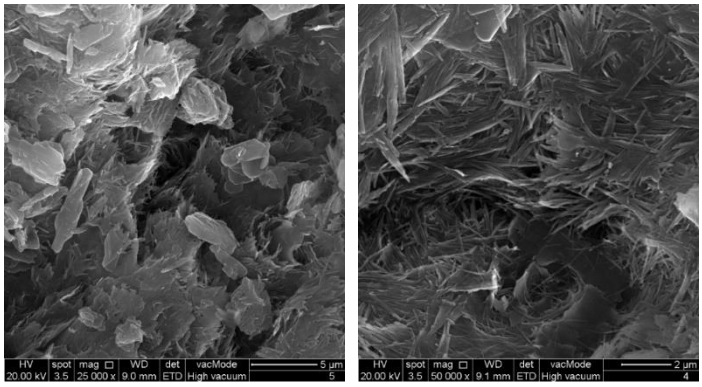
[359] demonstrated the decolourization of calcinated spent clay without further acid activation step almost remained constant after five repeated cycles, indicated that clay could be used repeatedly to adsorb unwanted compounds without affecting its performance. Low, Shamsuddin [361] had recovered the oil from the spent bleaching earth through acetone for energy generation and regenerating the bleaching earth. This process generated net earnings of $213,682 and had a payback period of 4 years under the best operating conditions. This procedure might be more financially feasible if greater amount of SBE were produced or if a centrally located facility was developed to handle spent bleaching earth from many plants. To conclude, in comparison to a virgin clay used as the benchmark, the bleaching capability of the regenerated clay demonstrated a similar bleaching effectiveness for microalgae oil.

Future research can investigate the role of reactivated clay in the removal of pigments from microalgae. Furthermore, the use of solvents with low volatility or high boiling point can be suggested as a modification to the microalgae lipid extraction method. Future research could compare different types of clay such as bleaching earth, Tonsil N, Fulmont clay, and Mexican clay. In addition, the relative removal ratios of fatty acids and chlorophyll by different proportions of clay and microalgae extracts also can be studied to evaluate the loss of fatty acids. Finally, the clay can be activated by combining physical and chemical treatments, such as the use of acid, alkali, ultrasound, mechanical treatments, or the incorporation of

nanoparticles on the clay for decolourization. In short, the performance of chlorophyll removal was not affected by the recycled components.

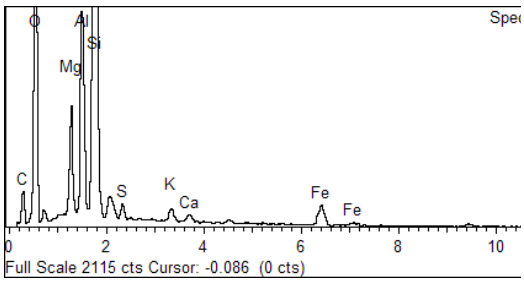


a **b** **c**

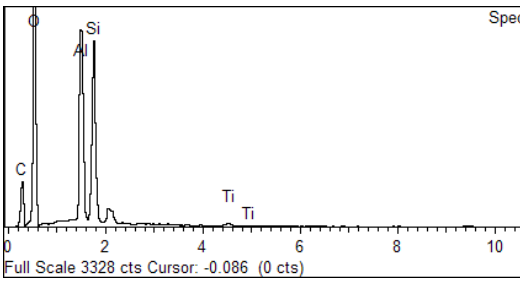


d **e**

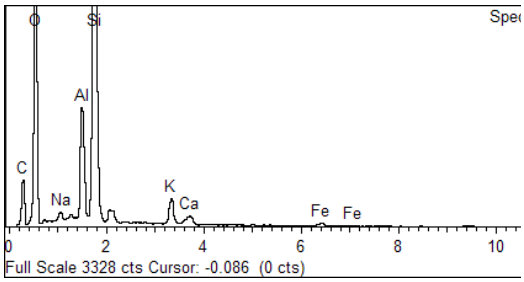
Figure 7.1 Field emission scanning electron microscope (FESEM) images at different magnifications which showed that all clay samples were made up of aggregate or cluster of particles: (a) bentonite clay, (b) kaolinite, (c) Fuller's earth, (d) bentonite clay at high temperature and (e) bentonite clay with ultrasound treatment.



a



b



c

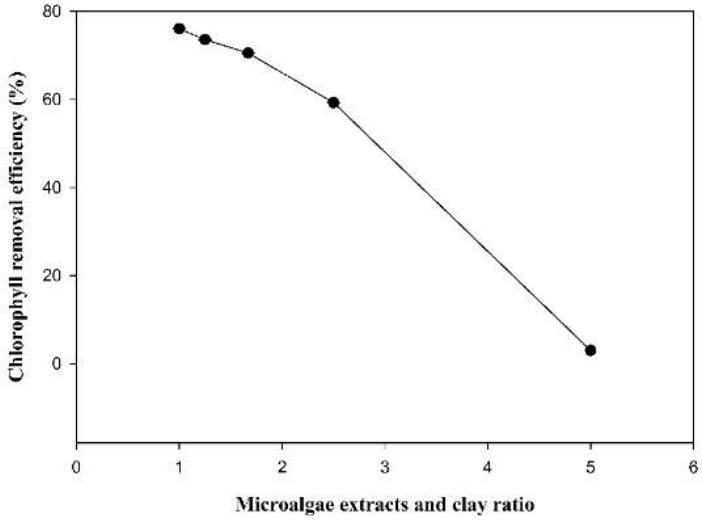
Figure 7.2 Energy-dispersive X-ray spectroscopy (EDX) spectra measured on clay samples: (a) bentonite clay, (b) kaolinite and (c) Fuller's earth.

Table 7.1 Elemental analysis of bentonite clay, kaolinite and Fuller's Earth.

Element	Weight (%)		
	Bentonite Clay	Kaolinite	Fuller's Earth
C	10.59	17.69	20.69

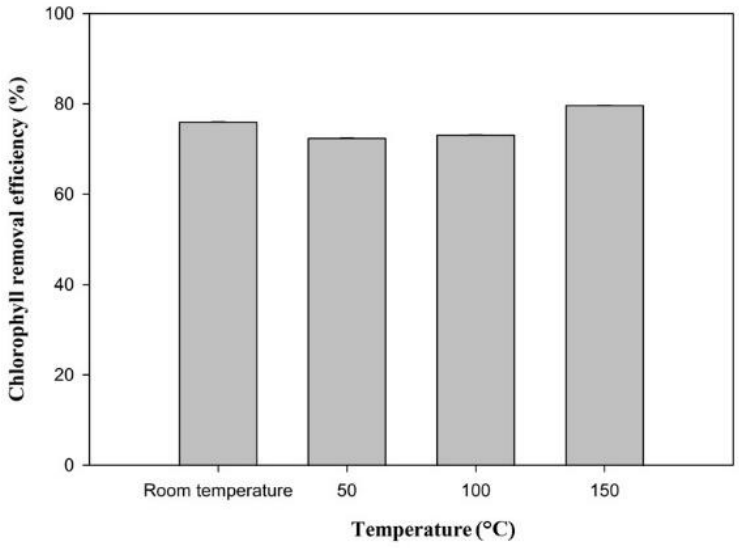
O	53.72	58.65	55.78
Mg	2.95	-	
Al	4.75	11.54	3.42
Si	23.84	12.13	17.45
S	0.90	-	
K	0.49	-	1.33
Ca	0.55	-	0.44
Fe	2.21	-	0.46
Na	-	-	0.43
Total	100	100	100

Ratio of microalgae extracts and clay powders vs chlorophyll removal efficiency

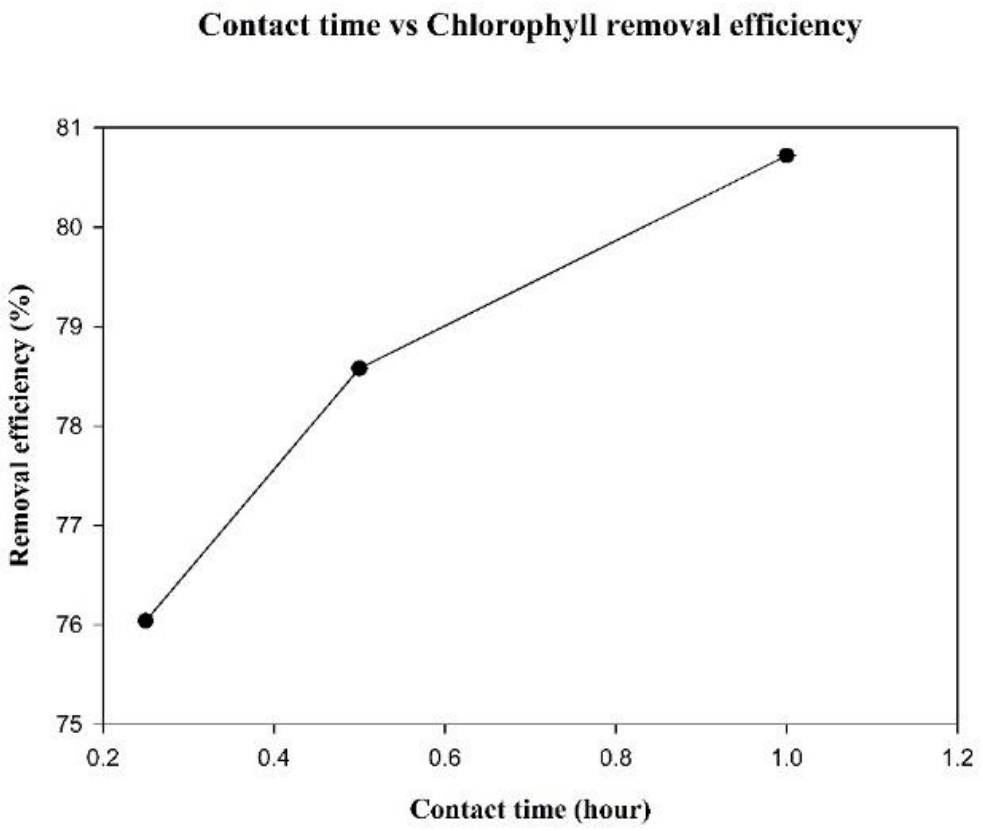


a

Temperature vs Chlorophyll Removal Efficiency



b



c

Figure 7.3 Effect of experimental parameters on the chlorophyll removal efficiency of bentonite clay: (a) clay load, (b) temperature and (c) contact time. The experiment was conducted in three replicates, with qualitatively similar results in each replicate and $p < 0.05$. Error bars represent standard errors.

Table 7.2. Chlorophyll removal efficiency of bentonite clay, kaolinite, Fuller's Earth and reactivated bleaching clay. The experiment was

conducted in three replicates, with qualitatively similar results in each replicate and $p < 0.05$.

Clays	**Chlorophyll removal efficiency (%)**	**Change in lipid content (%)**
Bentonite clay	76.04 ± 0.0212	-32.90
Fuller's earth	64.39 ± 0.364	NA
Kaolinite	50.28 ± 0.132	NA
Reactivated spent clay	**Chlorophyll removal efficiency (%)**	**Change in lipid content (%)**
Bentonite clay	70.35 ± 0.000205	- 42.26
Kaolinite	41.84 ± 0.423	NA
Fuller's earth	52.70 ± 0.139	NA

7.5 Conclusion

The surface area and porosity of the adsorbents was associated with the bleaching effectiveness of the clay. The finding reveals that bentonite clay was effective in removing the chlorophyll from the microalgae oil as compared to kaolinite and Fuller's earth. Optimization of the parameters showed that the removal efficiency increased with increased clay dosage,

longer contact time, high heating temperature and combination of ultrasound treatment. The optimum conditions were 1:0.8 (wt.%) microalgae extract to clay dose, room temperature and use of ultrasound treatment. The present feasibility study also proved the potential of reusability of the bentonite in the removal of chlorophyll from the microalgae lipid extracts. This is a significant waste management contribution to reduce and reuse the waste produced from edible oil industry and save production cost.

CRediT authorship contribution statement

Doris Ying Ying Tang: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Kit Wayne Chew**: Conceptualization, Methodology, Supervision, Writing – original draft. **Francesco G. Gentili**: Resources, Supervision, Project administration. **Tonni Agustiono Kurniawan:** Investigation, Writing – review & editing. **Young-Kwon Park:** Visualization, Validation. **Heli Siti Halimatul Munawaroh,** Data curation, Writing – review & editing. **Saravanan Rajendran,** Validation, Project administration. **Zengling Ma:** Supervision, Writing – review & editing. **Sarah Alharthi:** Formal analysis. **Walaa F. Alsanie**: Writing – review & editing. **Pau Loke Show**: Resources, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 8 CONCLUSIONS AND FUTURE WORKS

8.1 Conclusions

This thesis has developed a sustainable and environmentally friendly extraction methods for recovering and extracting biomolecules from the microalgae. Besides, this research work has developed an effective pre-treatment method to remove chlorophyll and at the same time, sustaining the lipid composition in the microalgae. The use of activated clay powders in the dechlorophyllization process has been attained. Lastly, the advancement of digital imaging to rapidly detect and estimate the chlorophyll content in the microalgae using smartphone camera also has been successful. The following is a detailed description of each research accomplishment:

a) **Triphasic partitioning of biomolecules from the microalgae:** The findings from analytical assays revealed that the biomass consisted of varied concentration of carbohydrates, protein, and lipids. The carbohydrates content in the mixed microalgae species was found to be 1.17 ± 0.04 mg/mL. Besides, protein content found in the study was 0.453 ± 0.003 g/L with the separation efficiency of 95.43%. The gravimetric analysis showed that the lipid content of microalgae was about 6.57% of the microalgae biomass. The lipid profile obtained from GC-FID showed that the highest peak was seen for C11 (undecanoic acid, 0.1870 mg/g), followed by C16 (0.0459 mg/g), C12 (0.0053 mg/g) and lastly C20 (eicosapentaenoic acid, 0.0012 mg/g).

The gas chromatography analysis results revealed that the fatty acid methyl esters profiles of the mixed microalgae species contain an abundance of saturated fatty acids (undecanoic acid, lauric acid) as compared to unsaturated fatty acids (palmitoleic acid, eicosapentaenoic acid). Phenolic compounds and antioxidant activity were also high, at 60.22 mg/L of phenolic compounds and 90.69% scavenging activity respectively.

b) **Extraction of lipase enzyme using liquid biphasic flotation:** The lipase production was optimized at 225 mg of microalgae biomass, 15 min flotation time with 125 cc/min as well as 99.8% ethanol and 275 g/L ammonium sulphate with volume ratio of 1: 1. Lipase recovery yield of optimal LBF system was 70.3% with separation efficiency of 82.0% and purification factor of 7.45.

c) **RGB in digital image processing to estimate the microalgae chlorophyll concentration:** The results indicated that acetone was a suitable extraction solvent. In comparison to CMYK model, RGB model offered a better correlation with high R^2. Among the three primary colours in RGB model, G colour chromate demonstrated the strongest correlation coefficient as compared to R and B colour. The predicted chlorophyll concentration by the regression and multilayer perceptron models and measured chlorophyll concentration by standard laboratory procedure were found to be significantly correlated.

d) **Pre-processing or pre-treatment of microalgae biomass in the chlorophyll removal:** The finding revealed that approximately 70% of the chlorophyll in biomass was removed. The oil yielded by chlorophyll-reduced biomass was orange-green colour. In the treated biomass, the proportion of the saturated fatty acids (lauric acid, behenic acid, palmitic acid and stearic acid) reduced and at the same time, unsaturated fatty acids (arachidonic acid) level increased. The lipid loss rate demonstrated by DMSO-sodium chlorite was satisfactory, which was around 13%. The proportion of polyunsaturated fatty acids with the DMSO-sodium chlorite treatment increased significantly by 92.5%. The pigment composition shifted toward carotenoids. The biochemical composition in treated biomass did not affect significantly as compared to untreated biomass.

e) **Activated clay as dechlorophyllization procedure:** Bentonite clay is found to remove chlorophyll effectively (76.04%) than kalininite and Fuller's earth. FESEM result reveals the presence of porous materials that facilitate adsorption. Additionally, the conditions of high temperature (79.69%) and ultrasound treatment (84.24%) demonstrate satisfactory removal efficiency of chlorophyll. Another promising finding is that chlorophyll can be adsorbed on the clays without affecting the lipid composition in the microalgae extract significantly.

8.2 Future works

This section focuses on current research gaps and future work to improve the downstream processing towards the biorefinery and commercialization of microalgae-based products in the future sustainably.

Firstly, future research should consider the potential effects of purification and characterization of lipases following extraction by LBF. The lipase enzyme can be characterized using SDS-PAGE. Isoelectric focusing and the enzyme activities toward various triglycerides and oils can be investigated. Kinetic constants of lipase enzyme also can be studied. Incorporation of gel column chromatography as a further purification step is proposed as a future work. Next, the chlorophyll concentration estimation model can be further enhanced utilising deep neural network and created a multiplatform application for all users. It is also possible to develop apps to be installed in the mobile device, utilizing the concept of “smartphone-based spectrophotometer” to estimate chlorophyll concentration. This is desirable for future work. Besides, in the dechlorophyllization process, future research should be devoted to the optimisation process to reduce the loss rate of total lipids below 10%, for example, temperature, duration, concentration, and volume ratio of solvents involved. Finding a balance between lipid loss and chlorophyll removal by optimising bleaching conditions is considered as an optimal bleaching concept. Lastly, future research should examine strategically on the cytological and reaction mechanism of chlorophyll

removal by comparing the morphological and ultrastructural alterations in control and dechlorophyllized microalgae cells.

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