Biorefinery of *Chlorella* sp. using integrated multiphasic systems for biofuel, feed and wound healing application

By

APURAV KRISHNA KOYANDE

Under the advisory of

Prof. Dr. Pau-Loke Show

A PhD thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering

JUNE 2021

Abstract

Microalgae have been explored as a sustainable alternative to fuel and feed on natural resources. Microalgae possess numerous advantages over their renewable counterparts such as soybean and palm oil. It does not compete with agricultural land or freshwater for food crop production, making it a potential biofuel source. However, commercialisation of microalgae biodiesel is yet to make a presence in the billion-dollar biofuel industry due to the bottlenecks. These include rigid microalgae cell wall, low biomass concentration in the harvested culture and high downstream costs. Therefore, a fossil fuel-derived concept of refinery can be introduced to microalgae to overcome as aforementioned challenges.

This project aims to focus on the algae downstream process for biorefinery applications. First, a novel biocomponent extraction method, named sugaring-out assisted liquid biphasic electric flotation (LBEF) system, for protein separation from *Chlorella vulgaris* was developed. High yield of proteins (69.66±0.86 %) was extracted from microalgae with a rapid and single-step process.

Following this, a multiphase integrated system that focused on the extraction of two or more biomolecules in microalgae was introduced. This system focused on simultaneous component extraction rather than conventional cascade approach. The system were incorporated in two different studies. First study aimed to extract two biomolecules (protein and lipid), whereas second study focused on a concurrent three biomolecules extraction approach. The parameters of this system such as volume ratio of ammonium sulphate and t-butanol, flotation air flowrate, flotation time, ultrasound pulse settings and pH were optimised to achieve a high recovery of biomolecules. Highest yield of protein, lipids and carbohydrates were observed at 96.59±8.15 %, 61.02±0.91 % and 52.69±1.90 %, respectively. Control run without flotation technique resulted in lower yield of proteins, lipids and carbohydrates at 25.33±3.50 %, 52.96±4.59 % and

32.44±0.29 %, respectively. Whereas, control run without flotation and cell-disruption technique had lowest yield of proteins, lipids and carbohydrates at 16.73±1.26 %, 51.13±6.27 % and 26.21±0.23 %, respectively. Besides, a large-scale set-up up to 10-15 times was tested out. Recycling ability of the chemicals involved in the extraction were presented. Up to 90 % of the alcohols and salt used in the experiment were recycled. Lastly, the extracted proteins from the multiphase integrated system were purified and its application in wound healing of human keratinocyte cells was investigated. Proteins were adsorbed on a gelatine-glutaraldehyde membrane. This membrane system was used to observe the wound healing of keratinocytes. The biocompatibility, cell adhesion, proliferation and wound scratch of human keratinocyte cells were studied and presented. Overall, multiphase integrated system presented in this project serves as a successful demonstration of microalgae biorefinery concept. The improved yield of biomolecules provide potential applications of microalgae in biofuel, food and medicine field industry. Future studies should focus on analysis of life-cycle cost and optimising the operational cost required for this whole biorefinery set up. The project presented in this thesis offers a platform for future biorefinery research and possible commercial large-scale utilisation.

Keywords

Microalgae; cultivation; integrated multiphase systems; liquid triphasic system; mammalian cell culture

List of Publications

1. **Apurav Krishna Koyande**, Chew Kit Wayne, Krishnamoorthy Ramababu, Yang Tao, Dinh-Toi Chu, Pau-Loke Show*. (2019) "Microalgae: a potential alternative to health supplementation for humans" *Food Science and Human Wellness*, 8 (1), 16-24. DOI: 10.1016/j.fshw.2019.03.001 [Accepted: 1 March 2019; IF: 2.455]
2. **Apurav Krishna Koyande**, Kit Wayne Chew, Jun-Wei Lim, Sze Ying Lee, Man Kee Lam, Pau-Loke Show*. (2019) "Optimization of protein extraction from *Chlorella Vulgaris via* novel sugaring-out assisted liquid biphasic electric flotation system" *Engineering in Life Sciences*, 19 (12), 968-977. DOI: 10.1002/elsc.201900068 [Accepted: 13 August 2019; IF: 1.934]
3. **Apurav Krishna Koyande**, Pau-Loke Show, Ruixin Guo, Bencan Tang, Chiaki Ogino, Jo-Shu Chang*. (2019) "Bio-processing of algal bio-refinery: a review on current advances and future perspectives" *Bio-Engineered*, 10:1, 574-592. DOI: 10.1080/21655979.2019.1679697 [Accepted: 3 October 2019; IF: 2.205]
4. **Apurav Krishna Koyande**, Vera Tanzil, Haridharan Murraly Dharan, Manivarman Subramaniam, Ryann Noel Robert, Phei-Li Lau, Ianatul Khoiroh, Pau-Loke Show*. (2020) "Integration of osmotic shock assisted liquid biphasic system for protein extraction from microalgae *Chlorella vulgaris*" *Biochemical Engineering Journal*, 157, 107532. DOI: 10.1016/j.bej.2020.107532 [Accepted: 7 February 2020; IF: 3.475]
5. **Apurav Krishna Koyande**, Kit Wayne Chew, Jun-Wei Lim, Man-Kee Lam, Yeek-Chia Ho, Pau-Loke Show*. (2020) "Biorefinery of *Chlorella sorokiniana* using ultra sonication assisted liquid triphasic flotation system" *Bioresource Technology*, 303, 122931. DOI: 10.1016/j.biortech.2020.122931 [Accepted: 27 January 2020; IF: 7.539]
6. **Apurav Krishna Koyande**, Kit Wayne Chew, Pau-Loke Show, Heli Siti Halimatul Munawaroh, Jo-Shu Chang*. (2021) "Liquid triphasic systems as sustainable

downstream processing of *Chlorella* sp. biorefinery for potential biofuels and feed production" *Bioresource Technology*, 333, 125075. DOI: doi.org/10.1016/j.biortech.2021.125075 [Accepted: 25 March 2021; IF: 7.539]

7. **Apurav Krishna Koyande**, Kit Wayne Chew, Sivakumar Manickam, Jo-Shu Chang*, Pau-Loke Show* (2021) "Challenges and Opportunities in the Emerging Algal Nanotechnology for the Generation of High-value Pharmaceuticals and Nutraceuticals" *Trends in Food Science and Technology*, 116, 290-302. DOI: 10.1016/j.tifs.2021.07.026 [Accepted: 22 July 2021; IF: 12.563]

Book Chapters:

8. **Apurav Krishna Koyande**, Bijal Mistry, Sze Ying Lee, Pau Loke Show*. (2020) "Flotation-assisted liquid biphasic system" *Liquid Biphasic System: Fundamentals and Applications in Bioseparation Technology*, p.105.
9. **Apurav Krishna Koyande**, Mohammad Ameer Abbas, Sze Ying Lee, Pau Loke Show*. (2020) "Electricity-assisted liquid biphasic system" *Liquid Biphasic System: Fundamentals and Applications in Bioseparation Technology*, p.187.
10. **Apurav Krishna Koyande**, Vishno Vardhan Devadas, Kit Wayne Chew, Pau Loke Show*. (2021) "Industrial Perspective of Industry 5.0" *The Prospect of Industry 5.0 in Biomanufacturing*, p.305.

Conference presentation:

11. **Apurav Krishna Koyande**, Kit Wayne Chew, and Pau Loke Show* (2019), August. POCER 1956: Utilization of Sugaring-out Liquid Biphasic Electric System for Protein Extraction from Microalgae. In Colloquium for Environmental Research (POCER 2019) 8-9 August 2019 Pulse Grande Hotel, Putrajaya, Malaysia (Vol. 8, p. 136).

Acknowledgements

Firstly, I would express my utmost gratitude to the grace of God who gave me courage to complete my PhD in these tough times. I am extremely grateful to my supervisor, Prof. Pau-Loke Show for his relentless guidance and persistent belief in me throughout my PhD journey. Thank you for always inspiring and motivating me to progress as a research student. Your absolute support, positive mindset and advice in the past few years are of vital importance to me.

I would also express my gratitude to my senior and mentor Dr. Kit Wayne Chew, who has always been by my side and guiding me throughout this marathon journey. Thank you for taking time off your busy schedule to nurture me.

My PhD journey would have been impossible without the financial support from my parents, who have supported me emotionally and mentally, too. I am very grateful for the grit shown by my family, sacrifices made by them and most importantly having the belief and faith in me especially when I am miles away from the home country. This degree is dedicated to my father's dream, who always wanted me to progress ahead of his qualifications. This journey has also become easier and possible with the constant support from my mother. I would also like to thank my late grandmother, who would always motivate and cheer for my little achievements.

Finally, I would like to thank my group mates, colleagues, friends and technical staffs for their support and assistance throughout.

Thank you all.

Declaration

The investigation presented in this thesis was conducted in the Department of Chemical and Environmental Engineering, University of Nottingham Malaysia (between July 2018–May 2021). Hereby, I declare that this work is purely based on my research findings and has not been submitted for any degree to any other institution. I declare that there are no known competing financial interests or personal relationships that could have appeared to influence the work reported in this thesis.

Abbreviations

ACN	Acetonitrile
NH_3	Ammonia
$(NH_4)_2SO_4$	Ammonium sulphate
ATPF	Aqueous two-phase flotation system
ATPS	Aqueous two-phase system
BSA	Bovine serum albumin
CR	Carbohydrates recovery
CO_2	Carbon dioxide
$Cu(NO_3)_2$	Copper (II) nitrate
mL/min	Millilitres per minute
°C	Degree celsius
DC	Direct current
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FTIR	Fourier transform infrared spectroscopy
GWP	Global warming potential
GTA	Glutaraldehyde
GGP	Gelatin-glutaraldehyde-protein
g	Grams
g/L	Grams per litre
h	Hour
H_2	Hydrogen

H_2S	Hydrogen sulphide
pI	Isoelectric point
ω-3 fatty acids	Linolenic acids
LR	Lipids recovery
LBEF	Liquid biphasic electric flotation
LBF	Liquid biphasic flotation
LCA	Life cycle analysis
LBS	Liquid biphasic system
LTF	Liquid triphasic flotation
L	Litre
LC-PUFAs	Long chain poly-unsaturated fatty acids
CH_4	Methane
µm	Micrometre
µmol/m^2/s	Micromoles per square metre per second
mL	Millilitre
NER	Net energy ratio
min	Minutes
nm	Nanometre
N_2	Nitrogen
OVAT	One variable at a time
OD	Optical density
O_2	Oxygen
PBR	Photobioreactor
PUFAs	Poly-unsaturated fatty acids
PR	Protein recovery

PSE	Protein separation efficiency
Rpm	Revolutions per minute
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
SS	Solvent sublation
SO_2	Sulphur dioxide
T-butanol	Tert-butyl alcohol
TPP	Three-phase partitioning
TEA	Tri-ethanolamine
U-LTF	Ultrasonication-assisted liquid triphasic flotation
UV-Vis	Ultraviolet-visible
V	Voltage
WHO	World Health Organisation
ω-6 fatty acids	α-linolenic acids
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Table of Contents

CHAPTER 1

1.1. Introduction

1.2. Research background

The Hunger Project, in November 2017, reported that out of 7.6 billion of the population in the world, 815 million do not have enough food to sustain themselves [1]. Amongst them, almost three quarters of the population is directly dependent on agriculture and similar activities [1]. The exponential population spurt threatens to reduce levels of food security as time progresses. According to *The Future of Food and Agriculture*, a report by UN's Food and Agriculture Organisation, "expanding food production and economic growth have often come at a heavy cost to the natural environment". It also stated that Earth is capable of fulfilling the demands of food security, however, the agricultural sector will require 'major transformations' to reach its full potential [2].

Microalgae has been utilised as food by humans for thousands of years and supplementation with help of microalgae will release the stress on intense resource demanding terrestrial food crops [3]. Algae are one of the oldest plants in the world as their first appearance dates back about 3.5 billion years ago. These photosynthetic species constitute of multi-cellular structure (macroalgae) of length up to 60 m and unicellular organisms (microalgae) with size as small as 0.2 μm [4]. Microalgae are extremely diverse group with estimated number of species ranging from 200,000 to 800,000 [5]. Some of these species are cultivated at industrial scale to extract bio-active compounds for human and animal consumption, cosmetics and bio-fuel industry [6].

Numerous species of microalgae are reported to contain similar amount of protein compared to the traditional protein sources like milk, soybean, egg and meat. However, extraction of protein from microalgae has various benefits in terms of nutritional value, efficiency and productivity. The protein yield from microalgae is reported at 4-15 tons/Ha/year compared to terrestrial crops production of 1.1 tons/Ha/year, 1-2

tons/Ha/year and 0.6-1.2 tons/Ha/year for wheat, pulse legumes and soybean respectively [7]. The production of terrestrial crops *via* agriculture accounts for roughly 75 % of total global freshwater [8]. Meanwhile, animal protein sources consume 100 times more water compared to plant sources for equivalent protein extraction [9]. Additionally, marine microalgae can be cultivated without freshwater and arable land further maximizing the resources required for additional terrestrial food crops production [7]. Moreover, due to the severe extreme environmental conditions and phototrophic mode of growth, microalgae are exposed to free-radical and high oxidative stresses. This has evolved the microalgae in developing natural protective system such as production of antioxidants and pigments (for example chlorophylls, carotenes and phycobiliproteins). These components are useful for human supplementation as they are not synthesised internally by individuals [10].

Moreover, microalgae species such as *Chlorella vulgaris, Scenedesmus* spp*.* and *Spirogyra* sp*.* are capable of accumulating lipids of its dry matter, with the range of 15-40 % and 70-90 % during normal [11–15] and extreme environments [16,17], respectively. Accumulation of lipid content affected by the stress levels imposed on the microalgae culture during cultivation [18]. When the microalgal culture medium contains a high carbon:nitrogen (C:N) ratio, N_2 will be exhausted in a shorter time, followed by accumulation of lipid [19,20]. A culture medium with high pH, high salinity, high temperature and limited N_2 source are some of the parameters for a faster lipid accumulation in microalgae [21]. Although biodiesel production from microalgal biomass is advantageous in various aspects, it is not as simple as its traditional counterparts as the processes involved complex extraction and purification. Numerous studies have been conducted to reduce the intricacies involved in harvesting, extraction and further biodiesel production. Abu-Shamleh and Najjar optimized mechanical harvesting by centrifugation and successfully reduced energy consumption by 90% [22]. Studies were

also conducted in harvesting of microalgae by flocculation, flotation and sedimentation [23–26]. Alhattab and Brooks conducted harvesting optimization by surfactant-aided dispersed air flotation and achieved 95% recovery [27]. Wahidin et al. optimized biodiesel production by one-step ionic liquid-microwave assisted process and achieved 42.22% biodiesel yield [28]. Rahman et al., optimized biodiesel from *Spirulina maxima* by two-step acid-alkali transesterification and resulted with 86.1% yield [29]. San Pedro et al., conducted a study to optimize biodiesel production by selecting suitable microalgae species and culture conditions [30].

1.3. Problem statement

Researchers have been studying possible alternative protein sources in addition to the conventional protein source due to the "protein gap" created in recent decades [31]. This is primarily a result of the rapid increase in population leading to escalation of the global food demand. In the long run, the existing agricultural methods might not be sustainable considering the negative impact on the environment (e.g. greenhouse gases, land clearing, nutrient run-offs *etc*.) [6]. Microalgae has been proposed to be one of the most reliable sources of protein as several of the microalgal species contain higher protein content compared to the common protein sources such as chicken, eggs and soybean [32,33]. Besides protein, microalgae also encapsulate lipids and carbohydrate, which are deemed as useful in food, feed and bulk chemical industries [34]. Furthermore, microalgae exhibits high photosynthetic ability [35] with a rapid growth rate and shorter harvesting cycle [36,37]. It also possesses high biomass density and higher degree of disease resistance [38]. It's ability to rapidly grow in the presence of carbon dioxide (CO_2), sunlight or any other light source and in salt water medium, qualifies microalgae as a potentially sustainable feedstock [39]. Since the culture of microalgae does not require arable land, it does not compete with nutrient-rich soil for agriculture [40] and

hence, are less susceptible to seasonal and weather limitations for cultivation [41]. There are more than 30,000 existing microalgae species in the nature. However, only less than 10 are produced commercially [12]. *Chlorella* is amongst the industrially cultured species for its lipid (used in biofuel), and protein (for food) metabolite [42]. This species is also favoured as it exhibits a fast growth rate [43].

Several methods of microalgae biomolecules extraction are available, which comprise of chromatography, supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), ultrasonication and ultrafiltration. However, these techniques are known to pose difficulties as they are laborious and time consuming, high in maintenance cost, non-environmental friendly and challenging for large scale extraction [44]. This poses a major obstacle for complete utilisation of microalgae's potential in the commercial industry. Extraction of multiple components from microalgae can tackle this bottleneck but cascade approach of biomolecules extraction results in higher expenditure compared to the output revenue. Thus, there is a need of an efficient approach for multicomponent extraction from microalgae such as refinery approach of crude oil.

1.4. Research objectives and contributions

The main aim of this PhD thesis is to develop an efficient biorefinery approach for extraction of multiple components from microalgae. Most of the currently reported downstream processes of microalgae are either expensive, involve multiple functional units or time-consuming. Herein, a novel integrated approach of multiphase extraction is presented. This approach involved a single unit while achieving a rapid product extraction. Furthermore, an application study is conducted to investigate the wound healing properties of the extracted proteins on human skin cells.

This research work can be broken down into four specific objectives:

1. **Optimisation of protein extraction from *Chlorella vulgaris via* a novel sugaring-out assisted LBEF system.** In this study, *Chlorella vulgaris* FSP-E was

cultivated and harvested. Protein from this microalgae biomass was extracted by liquid-liquid extraction. A sugaring-out method was employed with glucose solution and acetonitrile. The objectives are as follows:

- To study protein extraction via sugaring out LBEF method
- To optimise the parameters of LBEF system
- To compare the performance of optimised integrated system with control groups

The parameters of this study were optimised. This scope was thoroughly presented in Chapter 3 of this thesis. This research was published in Engineering in Life Sciences Journal. (**Apurav Krishna Koyande**, Kit Wayne Chew, Jun-Wei Lim, Sze Ying Lee, Man Kee Lam, Pau-Loke Show*. (2019) "Optimization of protein extraction from *Chlorella Vulgaris via* novel sugaring-out assisted liquid biphasic electric flotation system" *Engineering in Life Sciences*, 19 (12), 968-977. DOI: 10.1002/elsc.201900068)

2. **Biorefinery of *Chlorella sorokiniana* using ultrasonication-assisted liquid triphasic flotation (U-LTF) system.** In this study, *Chlorella sorokiniana* CY-1 was cultivated and harvested. Salting-out of this biomass was conducted. A resultant three-phase was observed due to interaction between ammonium sulphate solution and t-butanol. The objectives were as follows:

- To test out protein & lipids extraction via novel U LTF system
- To optimise the parameters of U-LTF system
- To compare the recyclability of the system with control studies

Protein and lipid from these phases was analysed and quantified. The parameters of this study were optimised. This scope was thoroughly presented in Chapter 4 of this thesis. This research was published in Bioresource Technology Journal. (**Apurav Krishna Koyande**, Kit Wayne Chew, Jun-Wei Lim, Man-Kee Lam, Yeek-Chia Ho, Pau-Loke Show*. (2020) "Biorefinery of *Chlorella sorokiniana* using ultra sonication assisted liquid triphasic

flotation system" *Bioresource Technology*, 303, 122931. DOI: 10.1016/j.biortech.2020.122931)

3. **Liquid triphasic systems as a sustainable downstream processing of *Chlorella* biorefinery for potential biofuels and feed production**. This research was published in Bioresource Technology Journal. In this study, *Chlorella sorokiniana* CY-1 was cultivated and harvested.

   The objectives were as follows:

   - To extract protein, lipids & carbohydrates simultaneously via novel U LTF system
   - To optimise the parameters of U-LTF system
   - To compare the recyclability of the system with control studies

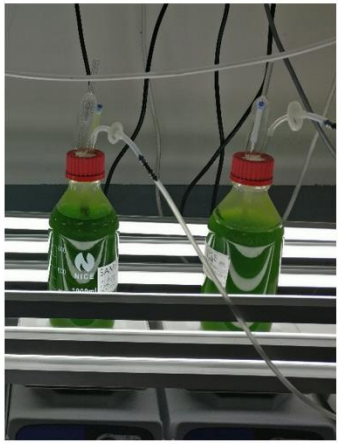
   Salting-out of this biomass was conducted. Addition of ultrasonication (cell-disruption technique) and flotation resulted in higher yield. Protein, lipid and carbohydrate from these phases was analysed and quantified. The parameters of this study were optimised. This scope was thoroughly presented in Chapter 5 of this thesis. (**Apurav Krishna Koyande**, Kit Wayne Chew, Pau-Loke Show, Heli Siti Halimatul Munawaroh, Jo-Shu Chang*. (2021) "Liquid triphasic systems as sustainable downstream processing of *Chlorella* sp. biorefinery for potential biofuels and feed production" *Bioresource Technology*, 333, 125075. DOI: doi.org/10.1016/j.biortech.2021.125075)

4. **Preliminary *in vitro* evaluation of proteins from *Chlorella vulgaris* coated on gelatine-glutaraldehyde membrane and its wound healing application**. In this study, proteins extracted from *Chlorella vulgaris* by previously mentioned integrated system were purified. The purified proteins were adsorbed on a gelatine-glutaraldehyde membrane. Wound healing properties of human keratinocyte cells by this membrane was studied and presented. This scope was thoroughly presented in Chapter 6 of this thesis. The objectives were as follows:

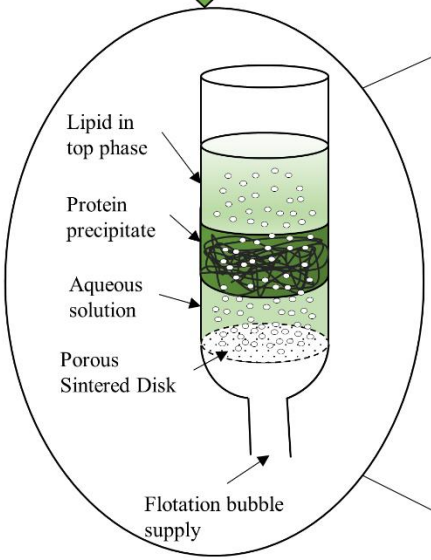
- To examine the cell proliferation with microalgae protein adsorbed on gelatine GTA membrane
- To study the adhesion capacity of keratinocyte cells on the membrane
- To investigate the wound healing rate of the protein on scratched HaCaT cells plate

The following section depicts the flow of research conducted in this project. Microalgae was cultivated by previously optimised protocol and harvested. The harvested biomass was dewatered and freeze dried to powder form. This powdered biomass was extracted by integrated multiphasic systems to obtain different biomolecules. The bottom of LTF cylinder was connected to a compressed air pump. The bottom aqueous phase of LTF system consisted of microalgal biomass dissolved in 100 mL of $(NH_4)_2SO_4$ salt solution in pre-determined concentration. The alcohol phase consisting of 100 mL of t-butanol was added to the aqueous bottom phase to form a three-phase system. During the operation of LTF system, an ultrasonication probe was submerged in the system for simultaneous cell disruption. At the end of each run, the three phases were collected *via* a tap located at the bottom of the equipment. The top phase consisted of lipid followed by protein concentrate in middle phase and carbohydrate in bottom phase. These phases were analysed for respective biomolecules content. Chapter 4 discusses on extraction of protein and lipid simultaneously whereas chapter 5 focuses on extraction of protein, lipid and carbohydrates from the three phases. The middle phase protein concentrate was purified and an application of the extracted protein from microalgae was conducted. Chapter 6 discusses on the application of microalgae derived protein on wound healing activity of human skin cells.

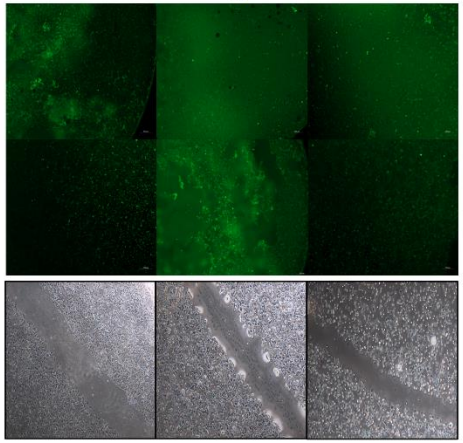
1.5. Flow diagram of the experimental methodology



MICROALGAE
CULTIVATION



EXTRACTION *via* LIQUID TRIPHASIC FLOTATION
Or
LIQUID BIPHASIC FLOTATION



WOUND HEALING PROPERTIES
OF MICROALGAE PROTEIN

CHAPTER 2

2. Literature Review

The booming world population, climate change, depletion of fossil fuels and increasing demand for food and energy are some of the concerns of the century [45]. The ever-increasing dependence on non-renewable fuel sources has sparked researchers' interest in securing alternative sustainable options when the fossil fuels run dry. The main external source of energy to Earth is from the Sun. The major part of this energy is harnessed by cultivating oil crops to convert solar energy into fuel by photosynthesis [46]. Researchers have investigated crops such as sugar cane for bioethanol; soybean, palm oil and rape seeds for biodiesel to secure the future demands *via* these renewable sources.

A second generation of biofuels were experimented by utilising the residual waste from agricultural biomass. The major drawback of these biofuels was the insufficient or irregular supply of the biomass required for fuel production [47]. In addition, these crops compete with the resources intended for food security reserves such as fertile land and freshwater. In the current scenario, only specific parts or compounds of these oil crops/plants are utilised for biofuel generation [48]. To overcome these bottlenecks, a biorefinery approach like the conventional petroleum refinery industry is introduced to ensure complete extraction of all the components in the biomass. To ensure economic optimisation in biorefinery, the raw material used is with reliable energy efficiency [49]. Examples of such material are biomass of crops, plants or microalgae [50,51]. Nonetheless, the void created by the drastic depletion of fossil fuels cannot be filled by traditional oil crops. Therefore, a third generation of biofuel derived from microalgal biomass has emerged in the past decade [18,52].

Compared to conventional oil-crops, microalgae can be cultivated on non-arable land or medium with saline, brackish water or wastewater [53]. Microalgae species are reported

to have high efficiency for photosynthetic conversion of sunlight as compared to the first and second generation biofuel sources [54]. Microalgal biomass can be directly converted to biofuel *via* four different techniques: biochemical conversion, thermochemical conversion, transesterification and microbial fuel cell. Selection of the technique is based on the specification and budget of the project, as well as the types and availability of the crude biomass feedstock.

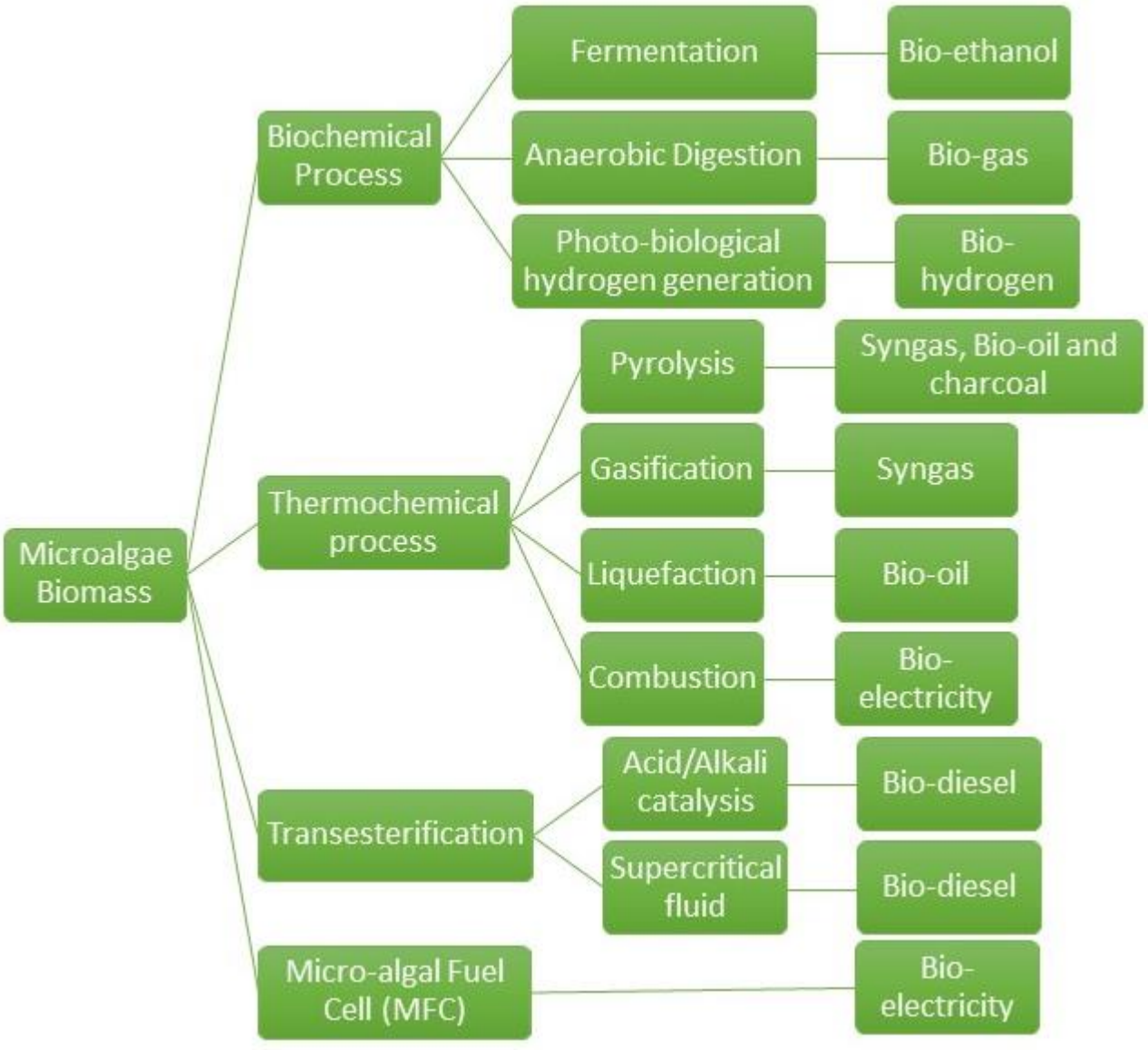


Figure 2.1 Bio-fuel production from microalgae biomass

The biochemical process involves biological conversion of microalgae feedstock into biofuels. Examples of biochemical process are fermentation, anaerobic digestion and photo-biological H_2 generation. Fermentation of microalgae to alcohol yields bioethanol. The fraction of microalgae that contains cellulose, starch and other organic components will be converted to alcohol *via* fermentation, in the presence of yeast [55]. Anaerobic digestion could also convert microalgal biomass into biogas. The biogas produced from microalgae possesses high energy content and recovery when compared to biodiesel production from microalgal lipids. The composition of biogas produced by anaerobic digestion consists of 50-70 % of CH_4, 20-30 % of CO_2, 0.1-0.5 % of H_2S, trace amounts of water, N_2, NH_3, H_2 and SO_2 [56]. Anaerobic digestion is preferred method as biodiesel production from microalgae involves high cost [57]. In photo-biological H_2 production, microalgae convert water into H_2 and O_2. However, the enzyme involved in biological H_2 production reaction (hydrogenase) is inhibited by O_2. Therefore, a temporal separation method has been proposed to separate H_2 and O_2 [58]. Melis *et al.* developed a temporal separation of both gases technique which involves two phases in microalgae *Chlamydomonas reinhardtii* [59]. In the first phase, microalgae fixed atmospheric CO_2 *via* photosynthesis to produce carbohydrates and O_2. This was followed by second phase, in which the culture medium was deprived of sulphur to inhibit photosynthesis and O_2 generation. In these conditions, hydrogenase enzyme favoured H_2 production.

Thermochemical conversion involves thermal decomposition of microalgal biomass to obtain various types of fuel.

Table 2.1 presents thermochemical conversion techniques of microalgae by pyrolysis, gasification, liquefaction and direct combustion. Pyrolysis of microalgal biomass involves thermal degradation of the microalgae under anaerobic conditions to produce biofuels with low to medium calorific value in a large-scale facility. On the other hand, gasification involves conversion of organic substrate to syngas by chemical biotransformation. Syngas is utilised either as an intermediate for production of various biofuels, or can be directly used in turbines and engines. Raheem *et al.* (2015) used a high temperature (703 °C) tubular furnace with a heating rate of 22 °C/min, biomass loading of 1.45 g with an equivalent ratio of 0.29 to obtain H_2 (41.75 mol%), CO (18.63 mol%), CO_2 (24.40 mol%) and CH_4 (15.19 mol%) [60]. Liquefaction is a hydrogenation process of bioconversion of wet microalgal biomass to biofuel under low temperature (300-350 °C), high pressure (5-20 MPa) and in the presence of a catalyst [61]. In contrast, direct combustion of microalgal biomass in aerobic condition yields heat, water and CO_2. The steam produced from direct combustion is used to generate electricity. The process efficiency can be enhanced by coupling this steam engine with the conventional coal-operated power plants [62]. Transesterification converts triglycerides found in microalgal lipids, in the presence of methanol, to fatty acid methyl esters (FAME) and glycerol. The process can be enhanced with addition of either acid or alkali catalyst.

Table 2.1 Thermochemical conversion technologies of microalgal feedstock.

Thermochemical conversion technology	Microalgae species	Process conditions	Results	Ref.
Slow pyrolysis	*Chlorella protothecoides*	1 g of sample, 5.5 mL stainless steel autoclave, 200-600 °C, 5-120 min	Maximum oil yield of 52 % at 500 °C for 5 min	[63]
	Nannochloropsis sp. after lipid extraction	1 g of sample, HZSM-5/sample (0/1-1/1), 300-500 °C, 10 °C/min for 2 h, N_2 at 30 mL/min	Maximum oil yield of 31.1 % at 400 °C. Achieved a higher heating value of 32.2 MJ/kg and lower O_2 content compared to direct pyrolysis	[64]
	Defatted and raw *Scenedesmus* sp. *and Spirulina*	100 g of sample, 450 °C at 50 °C/min, 2 h, N_2 as carrier gas at 100 mL/min	Observed a higher heating value in the range of 35.2-36.7 MJ/kg. Bio-oil yield in the range of 24-31 %	[65]
	Tetraselmus chui	2.4 g of sample, maximum temperature of 500 °C, 20 min with 10 °C/min, helium carrier gas at 50 mL/min in fixed bed infrared pyrolysis oven	The bio-oil obtained contains various alkanes, alkenes, aldehydes, amines, fatty acids and phenols. The bio-oil and biochar exhibited a high heating value of 28 MJ/kg and 14.5 MJ/kg, respectively	[66]
	Tetraselmus chui, Chlorella vulgaris, Chaetocerous muelleri, Dunaliella tertiolera	100 mg of sample, max temperature of 750 °C, 10 °C/min, helium carrier gas at 50 mL/min	Maximum bio-oil yield (43 %) for *Tetraselmus chui* at 500 °C	[67]
Fast pyrolysis	*Chlorella protothecoides*	200 g of sample, 4 g/min, 400-600 °C, N_2 carrier gas at 0.4 m^3/h, vapour residence time of 2-3 s in fluid bed reactor	Maximum bio-oil yield of 57.9 % at 450 °C. High heating value of 41 MJ/kg at low density and viscosity of 0.92 kg/L and 0.02 Pa.s with low O_2 content	[68]

	*Chlorella prototheco*ides and *Microcystis aeruginosa*	200 g of sample, 4 g/min, 500 °C, N_2 carrier gas at 0.4 m^3/h, vapour residence time of 2-3 s in fluid bed reactor	A higher (1.4 times) heating value of 29 MJ/kg of bio-oil than wood	[69]
Microwave-assisted pyrolysis	*Chlorella* spp.	30 g of sample, 6 g solid char as catalyst, 500-1250 W (462-627 °C), 20 min, N_2 carrier gas at 500 mL/min	Maximum bio-oil yield of 28.6 % at 750 W. High heating value (30.7 MJ/kg) of bio-oil	[70]
	Chlorella vulgaris	30 g of sample, 750-2250 W, 5 % activated carbon catalyst, N_2 carrier gas at 300 mL/min	Maximum bio-oil (35.83 wt%) and bio-gas yield (52.37 %) obtained at 1500 W and 2250 W, respectively. Yield is enhanced with activated carbon as catalyst	[71]
Hydrothermal liquefaction	*Chlorella vulgaris, Nannochloropsis oculata, Porphyridium cruentum* and *Spirulina*	3 g of sample, 75 mL reactor, 27 mL of distilled water,1 M Na_2CO_3 or 1 M formic acid, 350 °C for 1 h	A high heating value ranged from 22.8 to 37.1 MJ/kg with bio-oil yields in range of 25-40 %	[72]
	Dunaliella tertiolecta	7 g of sample, 100 mL stainless steel autoclave with magnetic stirrer, 70 mL distilled water, 0-10 % Na_2CO_3 as catalyst, 280-380 °C for 10-90 min	Maximum bio-oil yield of 25.8 % at 360 °C, 50 min and 5 % Na_2CO_3. High heating value (30.74 MJ/kg)	[73]
	Nannochloropsis sp.	4.27 g of microalgae paste (79 % water content), 200-500 °C, 60 min in 35 mL stainless-steel reactor	Maximum bio-oil yield of 43 % and the highest heating value of 39 MJ/kg at 350 °C	[74]
	Spirulina platensis	1.8 L reactor fitted with agitation impeller (300 rpm), 500-750 mL algal slurry with 10-50 % solids, 200-380 °C, 0-120 min, N_2 carrier gas with initial pressure of 2 MPa	Maximum bio-oil yield of 39.9 % at 350 °C, 20 % solids and 60 min	[72]

Microalgae are photosynthetic species that require sunlight to convert nutrients present in the medium (*i.e.* water) to bioactive components in their cell structure [75]. Due to the suspension nature of the medium, microalgae growth can be controlled and automated with better precision. Microalgae can be cultivated with three major sources: water, sunlight and CO_2. These resources are abundant and inexpensive. Conventional crops do not compete the resources required for cultivation of microalgae. Nevertheless, the microalgae culture medium needs to be nutrient-rich and contain various salts [76]. These nutrients can be obtained from household or industrial wastewater. Microalgae are able to grow and assimilate CO_2 under high CO_2 concentration condition, such as flue gas of a thermal power plant [37]. Coupled with high value added product output, microalgae portray promising potential as a sustainable source of energy for the future [77].

Microalgae are mainly composed of lipids, proteins, carbohydrates and traces of antioxidants and pigments. The constituents of different species of microalgae are presented in Table 2.2. However, microalgae cultivation and harvesting processes involves high energy consumption, high maintenance costs and are capital and labour-intensive. Therefore, optimisation of energy and expenditure for obtaining valuable products is crucial [78].

For example, the lipids extracted from microalgae can be utilised for biofuel production while proteins and whole biomass can be consumed as feed in livestock rearing and aquaculture. Additionally, carbohydrates obtained from microalgae can be fermented to produce bioethanol, used as an alternative carbon source to lignocellulose biomass or simple sugars in the bioethanol industry [79]. The unsaturated long-chain fatty acids extracted from microalgae exhibit important health benefits including potential anti-inflammatory and anti-carcinogenic effect on humans [80,81]. Apart from the three major fractions,

microalgae contain pigments such as chlorophylls, carotenoids, phycocyanin and astaxanthin [82], which are beneficial in pharmaceutical and cosmetic industry [83,84].

Table 2.2 Composition of different species of microalgae.

Microalgae species	**Composition (% dry matter)**			**Ref.**
	Protein	**Lipids**	**Carbohydrates**	
Anabaena cylindrica	43-56	4-7	25-30	[85]
Aphanizomenon flos-aquae	62	3	23	[4]
Chaetoceros calcitrans	36	15	27	[12]
Chlamydomonas reinhardii	48	21	17	[11]
Chlorella vulgaris	51-58	14-22	12-17	[86]
Chlorella pyrenoidosa	57	2	26	[12]
Chlorella protothecoides	NA	NA	50	[87]
Chlorella zofingiensis	NA	65.1	NA	[88]
***Chlorococcum* sp.**	NA	39.8-41.0	NA	[89]
Diacronema vlkianum	57	6	32	[90]
Dunaliela salina	57	6	32	[12]
Dunaliela bioculata	49	8	4	[14]
Euglena gracilis	39-61	22-38	14-18	[12,14]
Haematococcus pluvialis	48	15	27	[90]
Isochrysis galbana	50-56	12-14	10-17	[12]
Porphyridium cruentum	28-39	9-14	40-57	[12,14]
Prymnesium parvum	28-45	22-38	25-33	[14]
Scenedesmus obliquus	50-56	12-14	10-17	[11,15]
Scenedesmus dimorphus	8-18	16-40	21-52	[12,14]
Scenedesmus quadricauda	47	1.9	21-52	[14]
***Spirogyra* sp.**	6-20	11-21	33-64	[14]
Spirulina maxima	60-71	6-7	13-16	[12]
Spirulina platensis	46-63	4-9	8-14	[12]
***Synechococcus* sp.**	63	11	15	[11]
Tetraselmis maculata	52	3	15	[14]

Despite encasing numerous beneficial and high-value components, the current extraction of bioactive components from microalgal biomass only focused on a single product extraction. The following section discusses the current extraction techniques and bioprocessing in microalgae biorefinery.

2.1. Biorefinery of microalgae

Microalgal biorefinery approach is a process of obtaining energy and other bioactive components from microalgal biomass as a feedstock. Microalgal biomass is a great raw material for biorefinery approach as it can yield multiple components, such as food and energy, for uses in different industries. Microalgal biorefinery is a promising alternative to alleviate global warming caused by emission of polluting greenhouse gases such as CO_2 in the environment [91]. However, in the microalgae biorefinery, separation of different fragments without any significant loss of other components is crucial. This issue can be addressed by employing scalable, low-cost and energy efficient separation techniques [92].

Despite of portraying a huge potential, current industrial microalgal biomass production is roughly 15000 tons/year [46]. This is very low compared to the demands required in the industry. A major factor governing this low production rate is the high cost involved in the cultivation, harvesting and extraction of microalgae. Therefore, microalgae is currently only employed to extract high value niche products [93].

On the other hand, biofuel production is on the lower end of the spectrum due to the strict competition with fossil fuels. The price of biofuel does not necessarily have to be lower than its non-renewable counterpart. However, biofuel production needs to be performed at a lower energy expenditure. The current extraction technologies are not able to overcome the low-price

standards set by conventional fuel. The major stages of microalgae biorefinery involve upstream and downstream processing. The upstream process mainly consists of microalgae cultivation. The raw materials involved in the upstream process are nutrients, water, light and CO_2 [78]. The nutrients such as phosphorous and N_2 govern the growth of microalgae. An optimum amount of nutrient supply ensures a higher biomass production and shorter maturation period [94]. The source of illumination also affects the growth rate of microalgae. Several studies confirmed that illumination *via* artificial lighting such as LED is more effective than direct sunlight for microalgae cultivation [95,96].

The downstream process of microalgal biomass consists of harvesting, extraction and purification of the value-added products. Conventional extraction techniques are mechanical methods (such as bead beating and blending, high pressure homogenisation); ultrasound and chemical methods (such as solvent extraction), as well as freezing-thawing, autoclaving and supercritical fluids [97]. These processes are costly, complex and involve multiple steps. The economic burden incurred due to these processes is huge and extraction of various high-value products from microalgal biomass should be viable at industrial scale [79]. Microalgal biomass can be majorly divided into three fractions: oil, protein and carbohydrate. Figure 2.2 focuses on possible product streams to obtain numerous products from a single energy flow. The by-products or residual wastes obtained can be either recycled in the culture medium as nutrients or used to produce power in form of combined heat and power plant in the biorefinery.

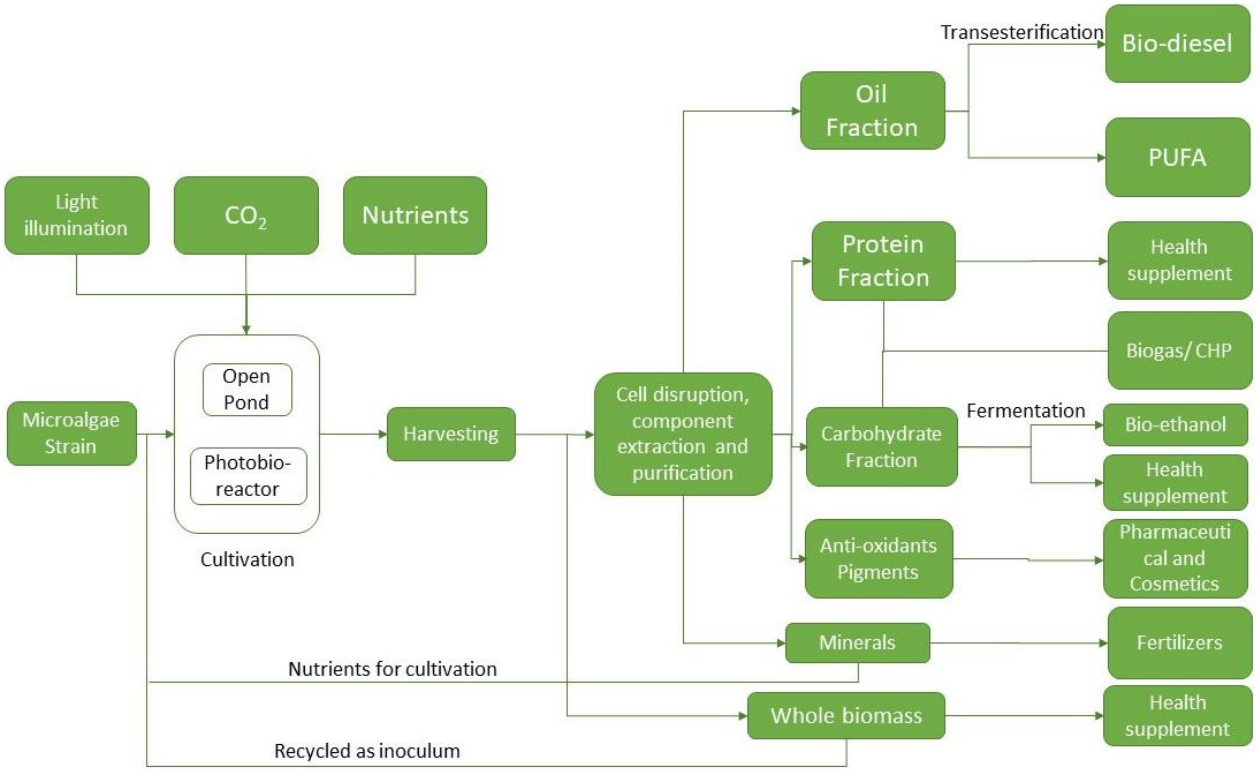


Figure 2.2 Microalgae biorefinery model.

2.2. Lipids fraction

Lipids from microalgae can be classified into two categories. The first type of lipid contains fatty acids with 14-19 carbon atom chains; while the second type contains more than 19 carbon atom chains. The former type is usually bio-transformed into biodiesel as it is a saturated fatty acid without the presence of any double bonds in the hydrocarbon chain. The latter type of

lipid is utilised in food industry as poly-unsaturated fatty acids (PUFAs) as it is an unsaturated fatty acid with the presence of double bonds in the hydrocarbon chain. Lipids productivity of microalgae is higher than that of traditional oil-crops. Table 2.3 presents biodiesel production and characteristics of various sources. It is evident that lipids from microalgal biomass are considered favourite for production of biodiesel.

Table 2.3 Biodiesel production and characteristics of various sources. Adapted from [86,98].

Type of source	Biomass oil content (wt%)	Yield (L oil/ha year)	Land required (m^2/kg biodiesel year)	Biodiesel production (kg/ha year)
Corn	44	172	66	152
Hemp	33	363	31	321
Soybean	18	636	18	562
Jatropha	28	741	15	656
Camelina	42	915	12	809
Rapeseed	41	974	12	862
Sunflower	40	1070	11	946
Castor	48	1307	9	1156
Oil Palm	36	5366	2	4747
Microalgae (Low oil content)	30	58700	0.2	51927
Microalgae (Medium oil content)	50	97800	0.1	86515
Microalgae (High oil content)	70	136900	0.1	121104

Lipids are commercially extracted from microalgae *via* solvents, ultrasonication, electrolysis or microwaves. These processes are energy-intensive, require the use of hazardous solvents, have low selectivity and need a high temperature [99–101]. There

are a few environment-friendly and simple solvent-free methods. One of the most promising technique is extraction of lipids from microalgae *via* super-critical CO_2 [102]. This method does not require hazardous solvents and is very selective to the non-polar lipid fraction of microalgae. This method also allows for further component extraction from residual cell debris [79]. Besides, CO_2 engaged in the process is recyclable. However, due to the super-critical nature of this technique, the capital and maintenance costs are levied on the production. The high costs can be neutralised by converting the remaining cell debris to fertiliser, fish feed or recycling back into microalgae cultivation for a higher productivity since this method is solvent-free.

The fragment of lipids required for biodiesel production is triglycerides (TAGs). TAGs are transformed to biodiesel by transesterification [103]. Transesterification is a reaction between methanol and microalgae lipids to obtain glycerol and FAME. In the mass balance of transesterification process, 3 moles of FAME and 1 mole of glycerol are obtained with 1 mol of TAG and 3 moles of methanol [104]. Transesterification can be accelerated during acid catalysis. Alkali catalysed reaction is 4000 times faster than acid-catalysed reaction [105]. Alkalis such as NaOH and KOH are usually employed in the alkali-catalysed reaction. However, saponification might happen due to the occurrence of free fatty acids in the TAGs. Therefore, a lipid-rich high-quality biomass is necessary to prevent saponification [106]. The upstream processing of microalgal biomass accounts for 65-70 % of the biodiesel production process. Acid-catalysed reactions have a slower reaction rate and lower yield compared to alkali catalysed reactions [107]. Due to the slower reaction rate and longer reaction time, acid catalysis is coupled with base catalyst in a two-step process [108]. In this two-step process, free fatty acids are converted to methyl esters *via* acid catalysts followed by conversion of residual triglycerides to methyl-esters by alkali-catalysts [109–112]. This process is beneficial as it utilises a low-quality feedstock. An increase in FAME yield up to 84 % was observed by using a wet microalgal biomass with 50 %(w/w)

water content [113]. The co-solvent used was methanol. Another study conducted utilised microalgae culture with a 90 % (w/w) water content to produce biodiesel using hexane and methanol in excess as co-solvents [114]. This process eliminates the extraction and produces FAME *via* direct transesterification. A similar study was successful in achieving a 97.3 % conversion rate of biodiesel by utilising *Chlorella vulgaris* with 71 % of water content [36].

PUFAs are parts of human cell membrane and function as an energy storing compound and cell signalling molecule [115]. Although humans can synthesise these lipids, some of the essential lipids must be obtained from diets. These lipids are known as glyco- or phospholipids. It contains two fatty acids chains and a polar head group. The most noteworthy group of glyco- or phospholipids is the long chain poly-unsaturated fatty acids (LC-PUFAs) [116]. LC-PUFAs are fatty acids comprising of three or more double bonds in a chain of 18 or more carbon atoms [117]. LC-PUFAs are generally classified into two families: linolenic acids (ω-3 fatty acids) and α-linolenic acids (ω-6 fatty acids). Among the two, ω-3 fatty acids have been reported to have numerous health benefits and incorporated in food products [118]. The essential fatty acids in ω-3 PUFA family are α-linolenic acid (ALA; 18:3), docosapentaenoic acid (DPA; 22:5), docosahexaenoic acid (DHA; 22:6) and eicosapentaenoic acid (EPA; 20:5).

Consumption of ω-3 fatty acids has shown effectiveness in prevention of various diseases such as arthritis, asthma, cancer, cardiovascular disorders, inflammatory bowel disorders, depression, schizophrenia and type-2 diabetes [117]. Food and Drug Administration (FDA) has recognized that ω-3 PUFA rich foods, particularly DHA and EPA, able to reduce risk of coronary heart diseases [119]. DHA plays an important role in development of infants, especially brain and retina [120]. Dietary supplementation with DHA is considered as a vital nutrient during pregnancy and breastfeeding as it actively contributes to

the development of nervous system of the young foetus. It also affects the cognitive function and visual acuteness of the child [121].

The main source of LC-PUFAs is fish and fish oil. Due to the potential contamination of fishes with toxins, there is an urgent need to explore alternatives. Over-exploitation of fishes, unpleasant odour and taste and oxidative instability are the reasons for this shift [117]. The primary producer of LC-PUFAs is marine microalgae. Marine microalgae contain these fatty acids in the purest form. These fatty acids can be accumulated and modified through multiple tropic food chains. The changes in the algal lipid content affect the dietary make-up of molluscs, shells, larvae and fishes [122]. Due to the rapid global warming and ocean acidification, there are reports of reduced supply of these fatty acids in the higher food chain [123]. Therefore, extraction of LC-PUFAs from microalgae is a promising alternative. Ryckebosch *et al.* reported that 0.8 g of fish oil is required to achieve daily ω-3 PUFA intake of 250 mg. The amount of oil required from *Nannochloropsis* sp. is around 1.3-1.4 g per day [124]. In view of the amount of microalgae required is less than half a tablespoon a day, this shows the potential of microalgae as an alternative to fish oil (especially for vegetarians/vegans).

2.3. Carbohydrate fraction

Microalgae are reported to contain carbohydrates as high as 50 % dry matter (Table 2.2). Carbohydrates secreted by microalgae majorly consist of monosaccharides such as glucose, fructose, mannose, galactose and polysaccharides (such as starch and cellulose). Glucose and starch extracted from microalgae are utilised in the production of biofuels such as biohydrogen and bioethanol [125]. However, polysaccharides majorly function as structural molecules and for storage purposes. Microalgal polysaccharides are reported to have the ability to activate macrophages, induce production of nitric oxide, reactive

oxidative species and various cytokines to modulate the immune system [126]. These macrophages can secrete chemokines and cytokines such as tumour necrosis factor (TNF-α) and interleukin (IL-6, IL-β). These compounds signal the inflammatory and immunomodulation reactions [127]. Tannin-Spitz *et al.* reported that the major function of cell-wall sulphated polysaccharide obtained from red microalgae *Porphyridium* sp. is to provide protection from external oxidative stress [128]. Matsui *et al.* reported that sulphated polysaccharides obtained from *Porphyridium* sp. have an ability to hinder the adhesion and migration of polymorphonuclear leukocytes, thus exhibiting anti-inflammatory properties [129]. The immunomodulating properties of sulphated polysaccharides from *Haematococcus lacustris* are evident as they stimulate the synthesis of pro-inflammatory cytokine from murine macrophages. Microalgal sulphated polysaccharides also exhibit a wide spectrum antiviral activity due to their interactions with surface molecules of virus cells. This not only inhibits the growth of host-type cells such as virus, but also blocks internal cellular fusion [130]. Therefore, sulphated polysaccharides have various medical applications owing to their pharmaceutical and therapeutic benefits including antitumor, anti-inflammatory, antioxidant and antiviral activities [83]. Table 2.4 summarises the pharmacological properties of microalgae.

Table 2.4 Pharmacological effects of microalgal carbohydrates.

Microalgae species	Type of carbohydrate	Pharmacological effects	Ref.
Chlorella stigmatophora	Crude polysaccharide	Anti-inflammatory, immuno-modulating	[131]
Chlorella vulgaris	Crude polysaccharide	Antioxidant	[132]
Gyrodinium impudicum KG-03	Sulfonated polysaccharide	Anti-viral	[133,134]
Haematococcus lacustris	Water-soluble polysaccharide	Immuno-stimulating	[127]

Phaeodactylum tricornutum	Crude polysaccharide	Anti-inflammatory, immuno-modulating	[131]
***Porphyridium* sp.**	Crude polysaccharide	Antioxidant	[128]
	Sulfonated polysaccharide	Anti-inflammatory	[135]
Rhodella reticulata	Extracellular polysaccharide	Antioxidant	[136]
Scenedesmus quadricuada	Crude polysaccharide	Antioxidant	[132]

Apart from pharmaceutical benefits, microalgal carbohydrates are majorly utilised for bioethanol production by fermentation. The microalgae are hydrolysed using acids or alkalis to break down carbohydrates in their sub-units. This process is known as saccharification [137]. Addition of water breaks down the polysaccharides into respective monomers. This process is crucial as it is a rate-limiting step in bioethanol production [138]. Hydrolysis of complex polysaccharides is mainly carried by chemical methods or enzymatic methods. Chemical hydrolysis or acid catalysed hydrolysis is rapid, and the chemicals used are cheaper than enzymes. However, the process creates lot of residual by-products that can potentially inhibit the next step of fermentation. On the other hand, enzymatic hydrolysis requires less energy but it is highly selective and thus require high amounts of enzymes for effective hydrolysis [139]. The monomers obtained after saccharification are fermented to using yeast, bacteria or fungi. Conventional process includes separate hydrolysis and fermentation or simultaneous hydrolysis and fermentation. However, these processes involve different micro-organisms and complex unit operations. Several studies have been conducted for production of bioethanol by hydrolysis and fermentation by microalgae itself [140,141]. Hirano *et al.* [142] reported that intracellular ethanol production is possible in *Chlamysomonas reinhadtii*. The culture is kept under anaerobic and dark conditions. This process also eliminates the expensive step of microalgae harvesting. However, the ethanol yield and production rate are lower than conventional two-step process [138].

Polysaccharides extracted from microalgae are utilised as stabilisers, thickening agents, emulsifiers, cosmetics, water-soluble lubricants, textiles, clinical drugs and in food and beverage industries [143]. The extracellular polysaccharides found in microalgae are beneficial with respect to bioprocessing as cell disruption is not necessary to extract these polysaccharides. Despite the multiple advantages of microalgal polysaccharides, it has not been successful in commercial market due to the cheaper alternatives like xanthan gum, agar, guar gum and carrageenan [6].

2.4. Protein fraction

Microalgal biomass consists of 40-70 % of proteins, although their quality is determined by its amino acids composition. Human body requires 9 essential amino acids which are not synthesised *in situ*. Conventional sources of proteins are meat, dairy, eggs, pulses and soybean. As compared to the conventional sources, microalgae are reported to be above-par source with respect to the essential amino acids composition. It has the potential to meet the protein requirements for the growing population. Microalgae use the least amount of land while producing a higher yield. A Life Cycle assessment conducted by de Vries *et al.* concluded that microalgae-based food products require less than 2.5 sqm of land per kg of protein; whereas, pork, chicken and beef require 47-64, 42-52 and 144-258 sqm of land per kg of protein, respectively [7,144]. Additionally, microalgae can be cultivated in a non-arable land and potentially use wastewater or seawater instead of fresh water. Their raw material requirements are lower than plant-based proteins such as pea protein, soybean protein [145]. Proteins are extracted from microalgae *via* various methods. The conventional extraction process utilised filtration or centrifuge to obtain the cellular components from soluble compounds in the liquid phase. These processes resulted in the loss of functional properties of the extracted proteins. On the other hand, utilisation of solvent extraction retains the functional properties of the proteins. In this

process, the soluble proteins are obtained by liquid-liquid extraction after cell-disruption [78]. The proteins are solubilised in organic solvents containing surfactants. The proteins are transferred from aqueous phase to the organic phase *via* electrostatic interactions between proteins and surfactants [146]. The parameters driving this process are pH, concentration and type of salts and organic solvents [79]. There has been an attempt to obtain proteins *via* super-critical CO_2 extraction which eliminated the use of toxic solvents [147].

2.5. Pigments (Carotenoids, chlorophyll)

Pigments are essentially molecules that absorb light from the visible spectrum. The wavelength which is not absorbed by these molecules is caught by human eye and therefore exhibit the corresponding colour. Pigments are used for a variety of products including food additives/colorants, aquaculture, pharmaceutical and nutraceutical products [148]. These pigments are currently produced on industrial scale *via* non-renewable synthetic sources such as petrochemicals, inorganic chemicals and organic acids. The raw materials and cost of production are cheaper with synthetic sources. However, there is a rising demand for naturally produced pigments due to the safety and environmental concerns associated with synthetically produced pigments [148–150]. Food industry is very keen on utilising natural pigments as food colorants owing to the harmful elements present in the synthetic sources [150].

Natural sources of pigment production are fruits, flowers, vegetables, insects and photosynthetic micro-organisms like microalgae and cyanobacteria [151]. Microalgae are photosynthetic species that produce various pigments depending on the species and their corresponding colours. For example, green microalgae contain chlorophyll; red and blue microalgae possess phycobiliproteins; yellow, orange and red microalgae synthesise carotenoids. Among the various sources, microalgae are

superior source of pigments due to their ability to synthesise natural pigments in a higher concentration compared to others [152]. Additionally, microalgae can be cultivated in brackish or wastewater with a high concentration of heavy metals. Microalgae consume nutrients present in the wastewater while reduce its dependence on chemicals and freshwater [55]. Furthermore, microalgae are 10 to 50 times more efficient in capturing CO_2 and sunlight for photosynthesis as compared to terrestrial plants [153]. Several studies have reported that *Scenedesmus* sp. and *Chlorella* sp. have the ability to tolerate 10-30 % of atmospheric CO_2 and hence have the ability to sequester large amounts of flue gas from nearby power plant [55,154]. Microalgal growth rates and productivity are higher than any plant systems [153]. Figure 2.3 depicts the production of carotenoids content in numerous natural sources.

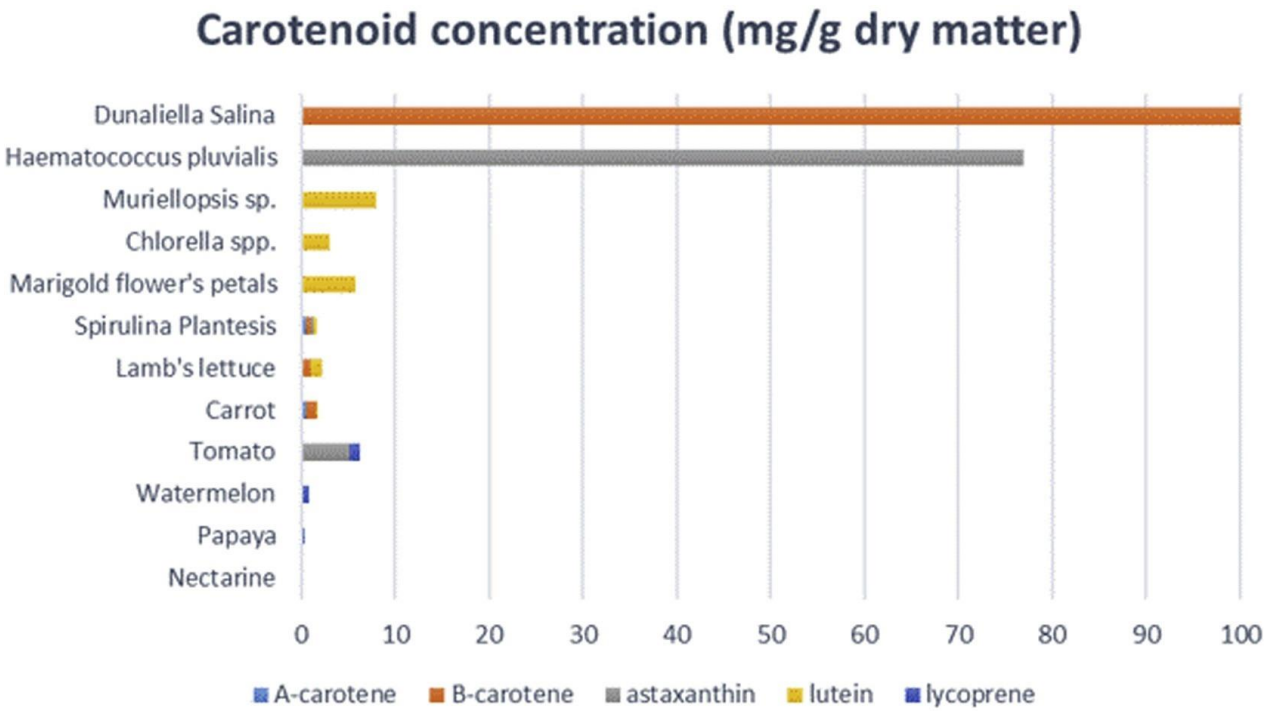


Figure 2.3 Carotenoid content of different sources including flowers, vegetables and microalgae. Adapted from [152,155].

Carotenoids are isoprenoid structured lipophilic pigments which are found in non-photosynthetic organisms, microalgae and higher plants [156]. These pigments have therapeutic effect on humans and animals due to their strong anti-oxidant properties to protect the organisms from oxidative and free-radical stresses [157]. Carotenoids act as pro-vitamin A in human body and are usually present in the range of 0.1-0.2 % of total dry matter of microalgae [4]. The global carotenoids market is estimated to be valued at USD 1.24 Billion in 2016 and projected to reach USD 1.53 Billion by 2021. However, most of the production incorporates chemical raw materials [149]. Carotenoids are classified according to their functional groups. Carotenoids with

only hydrocarbon groups are known as carotenes. Carotenoids with epoxy, hydroxyl and oxo groups are known as xanthophylls. With more than 400 identified carotenoids, only β-carotene and astaxanthin are industrially commercialised [11].

β-carotene is an important carotenoid pigment due to the presence of pro-vitamin A, an additive in multivitamin supplement and tablets. β-carotene is also applied as food dye on cheese, butter and margarine [11]. D. Salina is reported to contain up to 10-14 % of its dry matter as β-carotene [158]. Rodrigues *et al.* identified 24 types of carotenoids in *Phordium autumnale*. Amongst them, all-*trans*-β-carotene, all-*trans*-lutein and all-*trans*-zeaxanthin are the major contributors with concentration of 225.44 $\mu g.g^{-1}$, 117.56 $\mu g.g^{-1}$ and 88.46 $\mu g.g^{-1}$ of biomass, respectively [159]. Recently, studies have shown that β-carotene production can be enhanced *via* manipulating the culture conditions to high salinity, high light intensity, lower nutrient content and extreme temperature [160][161]. Optimising the culture medium conditions increases the production efficiency of β-carotene and 9-*cis* isomers [162]. 9-cis isomers of β-carotene have shown positive results on plasma lipids and thus have a possibility of preventing advancement of atherosclerosis in humans [163]. A Manfredonia study conducted on 640 individuals to evaluate the effects of β-carotene in cardiovascular health concluded that atherosclerosis diseases can be prevented with adequate addition of β-carotene and other antioxidants in diet, given that other external factors such as smoking, hypertension, dyslipidaemia and diabetes are under control [164].

Astaxanthin is a natural pigment and possesses powerful antioxidant properties compared to vitamin C, E or any other carotenoids like β-carotene, lutein, lycopene and zeaxanthin [86]. Astaxanthin is the second most important carotenoid pigment and mostly extracted from *Haematococcus pluvialis*, a freshwater green microalgae [165]. *Haematococcus pluvialis* is reported to contain astaxanthin about 4-5 % of their dry weight [157]. Apart from *H. pluvalis*, *Chlorella zofingiensis* also

produces astaxanthin in a low production rate. Under N_2 limiting conditions, *C. zofingiensis* produces hydroxyl radicals which are responsible for astaxanthin generation [166,167]. It is commercially marketed as a feed for salmon fishing industry [86]. Consumption of food rich in astaxanthin is promoted as a beneficial dietary supplement as its antioxidant strength is 100 times that of α-tocopherol [165,168]. Recently, several studies have been conducted to prove that addition of astaxanthin in diet of humans reduces inflammation, oxidative stress and improves the immune system of patients suffering from cardiovascular problems [169–171]. Kim *et al.* reported that consumption of *Haematococcus* astaxanthin resulted in prevention of oxidative damage in heavy smokers by suppressing lipid peroxidation [170]. Yoshida *et al.* reported that an increase in serum HDL and adiponectin in 61 non-obese subjects with daily dose of 12-18 mg of natural astaxanthin [172]. Table 2.5 illustrates phenolic and carotenoid activity with respect to different microalgae strains.

Table 2.5 Carotenoid and phenolic activity of various microalgae strains. Adapted from [173,174].

Microalgae species	**Carotenoid activity (mg/g DE)**	**Phenolic activity (mg GAE/g EW)**
Botryococcus braunii	2.10±0.07	1.99±0.17
***Chaetoceros* sp.**	1.9±0.2	11.9±0.28
***Chlorella* sp.**	0.3±0.04	8.1±0.16
***Dunaliella* sp.**	10.8±2.02	14.0±0.43
Dunaliella salina	3.8±1.30	19.3±0.70
Haematococcus pluviallis	1.89±0.05	1.23±0.06
***Isochrysis* sp.**	6.1±1.70	13.4±0.16
Nannochloropsis gaditana	3.0±0.24	32.0±0.57
Phaeodactylum tricornutum	6.3±0.10	16.8±0.33
Scenedesmus obliquus	0.44±0.06	1.94±0.16
***Tetraselmis* sp.**	4.6 0.90	25.5±1.50

Chlorophyll, a natural source of green pigments, is synthesised by all the photoautotrophic microalgal species and constitutes for about 0.5-1.5 % of the dry cell matter. The microalgae *Phordium autumnale* was also reported with two chlorophyll pigments namely chlorophyll-a (2.7 $\mu g.g^{-1}$) and chlorophyll-b (0.7 $\mu g.g^{-1}$)[159]. Chlorophyll consumption has shown a potential to increase bile secretion and further stimulate the recovery of liver [175]. It also possesses antioxidant, anticarcinogenic, antigenotoxic and antimutagenic properties [117]. Chlorophyll is a well-known detoxifying agent and a phytonutrient. It has a positive effects in human reproduction and improves metabolism of proteins, carbohydrates and lipids in humans [176]. Chlorophyll contains chlorophyllin (CHL), a sodium-copper-salt water-soluble derivative, which is readily absorbed by human body. Nagini *et al.* reported that addition of CHL in diet, has a potential to inhibit the progression of cancer as it targets multiple pathways of carcinogens and invades their cell cycle [177]. A study conducted by Das *et al.* successfully reported that introduction of CHL aided in slow progression of lung cancer in mice. The study further concluded that due to the ability of CHL to cross the blood-brain-barrier in mice, it will potentially be successful in human application [178].

Phycobiliproteins are water-soluble or hydrophilic protein complexes that capture light energy and thus assist in photosynthesis of cyanobacteria and other red microalgae [179]. The major producers of this pigment are *Porphydrium* sp.*, Spirulina* sp. *and A. flosaquae* [12]. Phycocyanin, a fluorescent blue-coloured phycobiliprotein, is extracted from *Spirulina* sp. as a natural dye. It is also used in popsicles, chewing gum, confectionery, wasabi, dairy products and soft drinks [11]. However, in nutraceutical industry, these pigments are marketed as anti-inflammatory, anti-oxidative, anti-viral, hepatoprotective and neuroprotective agents [6,117,180].

2.6. Vitamins

Apart from macronutrients such as proteins, carbohydrates and fats, the human body requires several micro-nutrients for survival. These micronutrients act as either co-enzymes or as an active electron/proton carrier in breakdown process of the macro-nutrients. One such group of important micro-nutrients are vitamins. Deficiency of vitamins in humans is responsible for various diseases such as beriberi, scurvy, rickets, methyl-malonic academia, *etc*. Vitamins play a major part in energy metabolism of humans. Depeint *et al.* reported that vitamin B_6, B_9 and B_{12} essentially regulate the mitochondrial enzymes in order to maintain the mitochondrial one-carbon transfer cycle of amino acid metabolism [181].

Algal food products are rich in vitamins. In 1990, Fabregas and Herrero conducted a study to determine the vitamin content of various microalgal species. They noted that microalgae contained high concentration of four vitamins; provitamin A, vitamin E, vitamin B1 and folic acid, compared to conventional food sources. They reported that *Dunaliella tertiolecta* was able to synthesise vitamin B_{12} (cobalamin), vitamin B_2 (riboflavin), vitamin E (tocopherol) and provitamin a (β-carotene). Moreover, *Tetraselmis suecica* was an excellent source of vitamin B_1 (thiamin), vitamin B_3 (nicotinic acid), vitamin B_5 (thenic acid), vitamin B_6 (pyridoxine) and vitamin C (ascorbic acid) [182]. *Chlorella* spp. are reported to contain vitamin B_7 (biotin) in high concentration. A study by Shim *et al.* concluded that around 9-18 % of *Chlorella* strains are rich source of vitamin B_{12} [183]. Watanabe *et al.* reported that although *Spirulina* species are capable of synthesising vitamin B_{12}, *Chlorella* species have better bioavailability [184].

Fruits and vegetables are poor sources of vitamin B_{12} (cobalamin) as it is neither synthesised nor required by plants [185]. This justifies the vitamin B_{12} deficiency among people following vegan and vegetarian diet [186]. Therefore, microalgal derived

foods provide an essential source of vitamin B_{12} for the individuals that follow these diets. Studies conducted have reported that several sea vegetables such as *Gracilaria changii, Himanthalia elongate* and *Porphyra umbilicalis* have similar levels of vitamin C compared to common vegetables like lettuce and tomatoes [187,188]. One species of brown microalgae, *Eisenia arborea*, reported to contain 34.4 mg of vitamin C per 100 g of dry matter. This value is closer to that of mandarin oranges [189]. *Macrocystis pyrifera,* more commonly known as Kelp, is reported to contain α-tocopherol at higher concentration that plant oils that are rich in this vitamin, like soybean, sunflower and palm oil [187,190]. Additionally, the levels of β-carotene in *Codium fragile* and *Gracilaria chilensis* was reported to outnumber that of carrots [187]. Although microalgae vitamin secretion is comparable to certain fruits and vegetables, implementation of biorefinery approach on microalgal bioactive component extraction deems it a superior source compared to conventional sources [79].

The fat-soluble vitamins extracted from microalgae need to be consumed with food items rich in lipids for effective absorption in the body. This co-relation is well understood, however, there is lack of literature data regarding edible microalgal biomass [190]. There are numerous studies that quantify the vitamin profile of microalgal species or study the potential of microalgal biomass as a functional food, although, there are no studies that incorporate both the aspects in one assessment [187,188]. Therefore, a comprehensive study which combines both these approaches need to be adopted for obtaining better insights on true potential of microalgae as a vitamin source [191].

2.7. Antioxidants

Human body continuously produces free radicals or reactive oxygen species (ROS) as a result of external stresses like smoking, chewing tobacco, excessive exposure to sunlight [192]. To counter these free radicals, antioxidants are synthesised by the

human cells. Additionally, human body has a balance between the oxidant to antioxidant ratio, any disturbance to this equilibrium will result in accumulation of free radicals. This phenomenon is known as oxidative stress. Oxidative stress is also related with numerous diseases such as diabetes, ageing, auto-immune disorder, cardiovascular disorder, atherosclerosis, rheumatoid arthritis, Alzheimer's disease, motor neuron disease and ocular disease [193]. Oxidative stress plays a pivotal role in cell and tissue damage which leads to chronic diseases such as cancer [194]. At the final stage of cancer, antioxidants are helpful in preventing further damage to human body due to carcinogenesis [195].

The production of the antioxidants in human body takes place in two pathways: *in-situ* and *ex-situ*. The antioxidants synthesised by human body *in-situ* or internally are known as endogenous antioxidants. Whereas the antioxidants that are consumed externally or *ex-situ* through food supplements are known as exogenous antioxidants. The endogenous antioxidants are synthesised by enzymatic or non-enzymatic pathways. Although, this paper emphasizes on the ability of microalgae to synthesise exogenous antioxidants. The antioxidants produced *ex situ* are also known as nutrient oxidants due to their nature. They constitute of carotenoids, flavonoids, trace metals (zinc and selenium), vitamin C and E, as well as fatty acids (ω-3 and ω-6 fatty acids) [193]. Table 2.6 illustrates PUFA composition of various freshwater and marine microalgae.

Table 2.6 LC-PUFAs composition of various microalgae species. Adapted from [196].

LC-PUFAs	**ALA**	**DHA**	**EPA**	**Total ω-3 PUFA**
***Chlorella vulgaris* (green)**	661±12	16±1	19±1	971±14

***Chlorella vulgaris* (orange)**	3665±1	80±1	39±1	4781±2
Diacronema vlkianum	14±1	836±41	3212±57	5407±146
Haematococcus pluvialis	3981±2	NA	579±6	5770±14
Isochrysis galbana	421±5	1156±40	4875±108	6461±153
Spirulina maxima	40±0.1	NA	NA	58±35

Microalgal biomass is superior source of nutritional antioxidants due to its higher production capacity compared to conventional plant-derived sources. Microalgae are also capable of producing multiple components in single species. For example, composition analysis on *Chlorella sorokiniana* revealed that the total carotenoid content was 0.69 % of dry matter. The α-tocopherol, β-carotene and lutein content were 112, 600 and 4300 µg/g of dry matter, respectively. These compounds possess high radical scavenging properties [197]. The antioxidant capacity of phycobiliproteins in *Phordium autumnale* was measured as 274 µmol trolox.g^{-1} of dry biomass weight [159]. It is also reported that antioxidant such as astaxanthin extracted from microalgae have greater antioxidant activity compared to α- carotene, β-carotene, lycopene, lutein and vitamin E. Although, it is lower than that from synthetically produced antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxytoluene (BHT) [198]. However, due to better awareness in the consumers, there is a huge demand for naturally sourced antioxidants. Moreover, as these compounds are included in pharmaceuticals, functional foods and beverages for human

consumption, extraction of natural antioxidants is gaining traction [4]. To conclude, the bioavailability of antioxidants extracted from microalgae is higher than synthetic sources and provide better protection [11,12].

2.8. Market trends, Life cycle analysis (LCA) and techno-economic analysis

In recent years, health related issues have been rising and there is a rising interest in consumption of 'healthy foods' or 'superfoods'. Superfoods are nutritionally dense functional foods which have added health benefits and may prevent or cure some chronic diseases. This has driven new research opportunities for evaluation of different sources for production of healthy functional foods [176]. Among the algal-derived products, the production of dried *Spirulina* spp. is the highest with around 12,000 tons per year, followed by *Chlorella* spp.*, Dunaliella salina, A. flosaquae, Haematococcus pluvalis, C. cohnii* and *Shizochytrium* with 5000, 3000, 1500, 700, 500 and 20 tons per year, respectively [117]. However, these values are very low compared to terrestrial crops such as palm oil whose production per year is 40 million tons [199]. The recently published Credence Research market report on algal products states that Compound Annual Growth Rate (CAGR) of algae-based products is expected to cross 5.2 % and the market value will stand at US$ 44.6 billion by 2023 [200]. Due to the rising demand of microalgae, particularly *Spirulina*, for applications in cosmetics and natural colorants, the CAGR of the global *Spirulina* market was expected to be around 10 % with an estimated value of US$ 2000 Mn by 2026. On the other hand, *Chlorella* ingredients market is anticipated to achieve a CAGR of 25.4 %, reaching US$ 700 Mn by 2022. The global market for natural sources of astaxanthin in aqua feed, cosmetics, food and beverages, and nutraceuticals also shows the potential of utilising microalgae to provide the needs of the respective market demand [117]. **Error! Reference source not found.** illustrates some of the examples and their benefits for human health.

Error! Reference source not found. Microalgae incorporation in food products.

Type of product	Microalgae species	Addition	Benefits	Ref.
Biscuits	*A. platensis*	1.63,3,5,7,8.36 % w/w	Nutritional and Techno-functional properties (protein, fibre and anti-oxidative content)	[201]
	A. platensis, Phycocyanin Extract	0.3,0.6 and 0.9 % w/w to wheat flour	Nutritional properties	[202]
	A. platensis, C. vulgaris, P. tricomutum and T. suecica	2 and 6 % w/w	Nutritional and Techno-functional properties (anti-oxidative activity)	[203]
	I. galbana	1 and 3 % w/w	Nutritional and Techno-functional properties (ω-3 PUFAs)	[204]
Bread	*A. fusiformis*	1 and 3 % w/w in the flour	Nutritional and Techno-functional properties (proteins and mineral content)	[205]
	A. platensis	11 % w/w in flour		[206]
	A. platensis (gluten free bread)	2-5 % w/w in flour		[207]
	A. platensis and O. amphibian	5 % w/w algal protein in flour		[118]
	Arthrospira sp.	2,2.5 and 3 % w/w in flour		[208]
	Dunaliella sp.	10 % w/w with algal biomass, biomass without β-carotene and biomass without β-carotene and glycerol.		[118]
	I. galbana, N. gaditana, S. almeriensis, T. suecica	0.47 % w/w in flour		[209]
Cookies	*C. vulgaris*	0.5, 1.0, 2.0 and 3.0 % w/w in flour	Colouring agent	[210]
	H. pluvialis	5, 10 and 15 % astaxanthin in flour	Nutritional and Techno-functional properties (antioxidative activity)	[211]
Extruded snacks	*Arthrospira sp.*	0.4, 1.0, 1.8, 2.6 and 3.2 % w/w	Nutritional and Techno-functional properties (proteins content)	[212]

Emulsions: oil/water	Green and orange *C. vulgaris* (after carotenogenesis)	2 % w/w	Techno-functional properties	[213]
	Green and orange *C. vulgaris* and red *H. pluvialis* (after carotenogenesis)	*C. Vulgaris*: 0.25-2.0 % w/w *H. pluvialis*: 0.05-2.0 % w/w	Colouring agent and nutritional properties (antioxidative activity)	[214]
Fermented milk	*A. platensis*	3 g/L	Nutritional properties	[215]
Frozen yogurt	*Arthrospira sp.*	2-8 % w/w	Nutritional properties	[216]
Pasta	*A. maxima,* green and orange *C. vulgaris* after carotenogenesis	0.5, 1.0 and 2.0 % w/w in flour	Nutritional and Techno-functional properties	[217]
	A. platensis	1-3 % w/w in flour	Sensory quality and nutraceutical potential	[218]
		5, 10 and 20 % w/w in flour	Nutritional and Techno-functional properties (antioxidative activity)	[219]
	D. salina	1-3 % w/w in flour	Nutritional and Techno-functional properties	[220]
	D. vlkianum and *I. galbana*	0.5, 1.0 and 2 % w/w in flour	Nutritional and Techno-functional properties (ω-3 PUFAs)	[221]
	S. platensis	5 and 10 % w/w in flour	Nutritional and Techno-functional properties (protein enrichment)	[222]
Probiotic yogurt	*A. platensis*	0.1-0.8 % w/w	Nutritional and Techno-functional properties	[118]
Processed cheese	*Chlorella* sp.	0.5 and 1.0 % w/w	Nutritional and Techno-functional properties	[223]
Vegetarian food gels	*A. maxima* and *D. vlkianum*	0.1- 1.0 % w/w	Nutritional and Techno-functional properties (antioxidative activity, ω-3 PUFAs)	[224]
	A. maxima, C. vulgaris, D. vlkianum and H. pluvialis	0.75 % w/w		[225]
	A. maxima and *H. pluvialis*	0.75 % w/w		[226]
Yogurt	*Chlorella* sp.	Powder extract: 0.25 % w/w, Liquid extract: 2.5-10 %	Nutritional and Techno-functional properties	[227]

Microalgal biomass encompasses high value products while utilising natural and anthropogenic resources. The potential of microalgal biomass of producing high value-added products has grabbed attention of various research groups involving biofuels, food and feed as well as pharmaceuticals. These traits deem microalgae feedstock as a suitable candidate for exploitation *via* biorefinery approach. However, before further research is conducted for potential industrialization, a thorough LCA is necessary. LCA quantifies all the resources that are required in microalgae cultivation, harvesting, extraction and purification and calculates the emissions and its effect on nature from the same process. Additionally, economic analysis of the whole biorefinery approach is crucial to understand the feasibility of microalgae as a feedstock. These tools provide an understanding of current scenario and generate various pathways to achieve commercial industrialisation of microalgal biorefineries.

Evaluation of LCA is conducted on the basis of two indicators namely global warming potential (GWP) and net energy ratio (NER). GWP is quantified by the amount of CO_2 emitted per unit of energy. Ideally, all the greenhouse gases are considered for this quantification, but literature data is limited to CO_2 emissions. Positive result of biofuel production from microalgae is however limited to hydrothermal liquefaction at carbon credit of -220 g $CO_{2\text{-eq}}MJ^{-1}$ compared to conventional diesel with carbon credit of +15 g $CO_{2\text{-eq}}MJ^{-1}$ [228]. NER is evaluated based on the total energy flow of the process. It is the ratio between energy required to obtain the final products from microalgae and the total energy stored in the final product. LCA has been carried out in various studies but have been limited to biofuel or bioenergy production from microalgae. Jorquera *et al.* [229] conducted a LCA on biomass production of *Nannochloropsis* sp. and evaluated the NER for three different cultivation setups. The NER values were obtained as 8.34, 4.5 and 0.2 for open/raceway ponds, flat reactors and tubular reactors. However, this study was only based on biomass cultivation and no further product extraction. On the other

hand, Tredici *et al.* [230] conducted LCA of *Tetraselmis suecica* cultivation with harvesting for biomass production. The study compared NER of flat panel bioreactor with and without a photovoltaic panel. The NER of bioreactor with photovoltaic panel was 1.73 compared to 0.82 without the external renewable energy supply. However, these values were still not sufficient compared to NER of 3.71, 4.11 and 7.57 of soybean, corn and cassava, respectively.

Recently, Bennion *et al.* [231] studied NER values of microalgae biofuel production from cultivation until transportation of biofuel to the fuel station. The NER values in this study ranged from 0.44 to 2. Although, these values were high, but they are not sufficient compared to NER value of 5.55 of fossil fuels. However, the NER values of biofuel production from microalgae fluctuate due to different system boundaries and are not comparable to the conventional fossil fuel NER values. Chowdhury *et al.* [232] conducted LCA on four scenarios based on energy production from microalgae by utilising dairy waste as a substrate. The four cases include anaerobic digestion, biodiesel production, pyrolysis and enzymatic hydrolysis. These scenarios resulted in NER values of 0.35, 0.48, 0.50 and 0.68, respectively. The authors concluded that production of biofuel alone is not feasible and thus biorefinery approach is necessary. A LCA study conducted by Soh *et al.* [233] based on energy consumption, green-house gas emissions and potential of eutrophication concluded that optimising extraction from a single fraction of microalgae does not result in positive environmental outcome. Microalgae feedstock post-lipid extraction should undergo further processing to extract valuable niche components such as pigments and PUFA while the starch fraction should be digested anaerobically. This will lead to much pleasant outcome rather than single product extraction.

Apart from LCA, economic feasibility of microalgae biorefinery is crucial to attract commercial industrialization. Hoffman *et al.* [234] conducted a comparative economic feasibility study of biodiesel production between Algal Turf Scrubber (ATS) and Open

Raceway Ponds (ORP). According to this study, the biodiesel production cost from ATS and ORP were calculated at $8.34 and $6.27 per gallon of biodiesel, respectively. Although, these prices are not providing positive economic feasibility. Dasan *et al.* [235] conducted a study of three different cultivation systems to obtain biodiesel and other by products from different fraction of microalgae feedstock. The cultivation system studied were open pond/raceway pond, bubble column PBR and tubular PBR. This life cycle study was based on production of 100,000 kg of biomass for 340 days of the year. The study concluded that capital cost involved in tubular and bubble column PBRs were higher than the operating cost ranging from 47.54-86.18 %. However, open ponds cultivation system requires 45.73 % of total cost for operation and maintenance. The study analysed production of bioethanol as a by-product, however the complex and costly processes involved in bioethanol production do not favour the economic profitability. On the contrary, a biorefinery economic assessment conducted by Lam *et al.* [236] predicted that the highest total revenue generated from microalgal biomass is around €31 per kg of dry weight compared to the production cost of €6-7 per kg of dry weight. Although, these values can only be achieved when the downstream processing costs are minimized. Therefore, simpler and cost-effective downstream processing techniques should provide economic feasibility of microalgae biorefinery.

2.9. Challenges and future prospect

The techno-economic evaluation concluded that with the existing downstream process techniques, the microalgae biorefinery approach is not sustainable and feasible. The major hurdle faced by microalgae cultivation process is the limited biomass concentration in the matured algae culture. The maximum biomass concentration in the autotrophic microalgae culture is limited to around 3 g/L compared to 30-100 g/L biomass concentration of heterotrophic bacteria. Microalgae cultivation is also expensive compared to the bacteria fermentations due to the utilisation of photobioreactors (PBRs)

equipped with artificial light for optimum cultivation parameters. The low biomass concentration of microalgal culture coupled with high downstream processing costs (around 40 % of total cost) hinder the success of biorefinery approach for effective extraction of all valuable components from microalgae. Gifuni *et al.* [237] analysed various studies conducted on microalgae biorefinery and concluded that cascade extraction was most suitable approach for effective utilisation of microalgae components. Various studies conducted using cascade extraction utilised a novel approach of extracting high-value added components such as lutein, astaxanthin and carotene followed by recovery of other by-products such as proteins and carbohydrates [238–241]. In this approach, the costs of microalgae cultivation and extraction are offset by the high-value pigments while extraction of remaining fraction can be profitable. Ansari *et al.* [242] conducted a biorefinery study of microalgae by extracting proteins with aqueous extraction technique which was preceded by extraction of high-value products such as pigments and PUFA. The study was conducted with cascade extraction of proteins followed by lipids and carbohydrates. Utilisation of mild liquid-based extraction resulted in limited damage to other fractions. This study concluded that recovery of maximum number of products from microalgae is dependent on the severity of the extraction technology and utilisation of wet microalgae paste which reduces the drying costs.

Despite the high market value, production of algae-based bulk products presents few hurdles. The existing large-scale facilities distribute their produce to aquaculture industry, animal feed industry or for the production of bioactive components [145]. Individual governments and regulatory authorities hamper the circulation of new microalgae products due to their complex rules and regulations on novel food products [243]. This has been a major obstacle in the potential growth spurt of commercially large-scale distribution of microalgae food products. There’s a need for targeted nutrition educational programmes for young individuals to convey the importance of microalgae

in human diet [244]. Attracting attention of investors for starting up a new facility is difficult as microalgae products do not have a proven record of high market demand compared to traditional terrestrial crops especially as food products. The cultivation and down-stream processing of traditional protein based on terrestrial plants is optimised throughout the years as opposed to microalgae protein-based food products. Therefore, further research in cultivation and processing are necessary to obtain a sustainable and profitable market for microalgae food products. The investors however look for long-term record of high market demand and high market value to risk financing in a new venture [118]. A study conducted by Ruiz *et al.* estimated reduction in cultivation and bio-refining costs up to 10 times per kg of biomass when the facility was upgraded from 1 hectare to 100 hectare in size [245]. However, such upgrades are not easy to execute. The biomass composition, a critical factor in its integration in food products, is driven by microalgae species and the cultivation conditions [34,55]. The capital-intensive steps are that of dewatering and harvesting. These steps drive the economics of the final product, however, the size of the plant and cultivation medium play an important role as well [246].

Current large-scale open-pond or lagoon microalgae cultivation and biomass production are based on harvesting microalgae from natural habitations [145]. They are cheaper to install and easy to run; however, they have high chances of predator contamination, irregular growth due to varying light and temperatures [247]. The future of microalgae production might be dependent on recently developed compact large-scale photobioreactors (PBRs). These PBRs can be operated at optimised parameters with minimum risk of contamination. However, they are expensive and in small-scale currently [248]. The scale-up of such systems is hindered by inefficient use of light by the microalgae. Recent studies have overcome this issue by designing special diodes and optical fibres to efficiently provide internal illumination to the PBRs [249,250]. Despite

all these issues with scale-up of PBRs, extraction of high value-added bio-components and nutraceuticals is still feasible with the current PBRs. However, it is too small and unprofitable for biofuel production [117].

Addition of bioactive components extracted from microalgae to commonly consumed food products can ensure nutritional benefits to majority of the population. Recently microalgae cells have been used as ingredients in various food products including biscuits, cookies and pasta. Gouveia *et al.* and Raymundo *et al.*[213,214] reported promising changes in the anti-oxidizing activity of food emulsions when certain microalgae species were infused in it. Incorporation of microalgae with dairy products has been successful as well [216]. It is reported that addition of *Arthrospira* spp*.* stimulates probiotic growth in fermented milk and yogurt [215]. The presence of vitamins, minerals and other trace metals in microalgae enhances the growth of probiotic bacteria [215,216]. Cookies and biscuits on the other hand are much simpler products to deliver bioactive components of microalgae. They have higher acceptance in general population due to their appearance, taste, texture and are easier to store and transport. There have been successful attempts of adding microalgae to pasta. Fradique *et al.* reported that microalgae added pasta presented very appealing colours and had similar appearance to pasta cooked with vegetables [217]. The use of microalgae enhances the sensory and nutritional quality of the pasta. Microalgae, if utilised to its full potential, can benefit human population immensely in the long run. It is useful in many ways from production of biofuels, animal feed, human food products, cosmetics, nutraceutical and pharmaceutical industry. Although, it is expensive to cultivate if only one product is extracted. Until today, various studies have been conducted to conduct the biorefinery approach on microalgae. Table 2.7 summarises an overview of the studies conducted till date. Although, for sustainability and profitability of microalgae cultivation, further research in an integrated

biorefinery approach is required which will extract multiple products including biofuels, pigments, PUFAs and antioxidants [79].

Table 2.7 List of biorefinery studies conducted on microalgae.

Feedstock	Extracted compounds	Ref.
Dunaliella tertiolecta	Lipids such as beta-carotene, fatty acids and phytosterol followed by pyrolysis to obtain char and bio-oil from defatted biomass	[251]
Isochysis galbana	Polar lipids and carotenoids such as fucoxanthin	[252]
Nannochloropsis gaditana	Proteins, carotenoids and biodiesel	[253]
***Nannochloropsis* sp.**	Lipids fraction such as carotenoids and fatty acids followed by bio-H_2	[254]
***Scenedesmus* sp.**	Amino acids with biogas	[255]
Defatted algal biomass	Short chain carboxylic acids and bio-H_2 production from algal biomass post lipids extraction	[256]

This section briefly discussed the importance of microalgae, the biomolecules secreted by it and its techno-economic and future prospects. The potential of microalgal biomass to produce high-value bioactive components enables it as a promising raw material for bioprocessing. Thus, in this thesis, a preliminary study incorporating protein extraction was followed by biorefinery approach in the next two studies. The biorefinery study includes Liquid Triphasic Flotation (LTF) system with simultaneous extraction of protein and lipid. To prove the potential of LTF system, the second biorefinery study examined simultaneous extraction of protein, lipid and carbohydrate from microalgae. After successful demonstration of biorefinery approach of microalgae bioprocessing, an application of purified proteins was conducted. The purified protein extracted by LTF system was adsorbed on gelatine-glutaraldehyde (GGP) membrane and its wound healing activity on human keratinocyte cells was investigated. The following sections of this thesis discuss these studies and future improvements are also listed. The LTF system proposed in this thesis has the potential to transform and upgrade the bioprocessing industry in the upcoming decade.

CHAPTER 3

3. Optimisation of protein extraction from *Chlorella vulgaris via* a novel sugaring-out assisted LBEF system

3.1. Introduction

Microalgae are photosynthetic unicellular organisms that are capable of rapid growth and survival in harsh conditions [257]. Compared to vegetables and other sources of proteins, microalgal biomass contains a high amount of protein. Microalgae species of *Spirulina* and *Chlorella* are noted to contain more than 70 % of their dry matter as proteins [40]. Due to such high protein content of microalgae, their biomass has attracted attention by various industries such as poultry, aquaculture, animal feed, *etc*. World Health Organisation (WHO) also recommends the consumption of microalgal biomass of *Chlorella* species in human diet due to its complete essential amino acids profile [258]. Protein extraction from microalgae is currently carried out *via* expensive separation processes such as crystallisation, column chromatography, ultrafiltration, membrane separation and precipitation. These conventional processes are time-consuming, complicated, require high maintenance cost and are difficult to scale up.

On the other hand, liquid biphasic flotation (LBF) is a novel upcoming technology that is capable of replacing conventional technologies [259]. LBF system is a combination of solvent sublation (SS) and aqueous two-phase system (ATPS). In LBF system, the air bubbles provided at the bottom of the set-up adsorb the bioactive components of the bottom phase. The bioactive components will be transported to the top phase by the rising gas bubbles. This novel method has been utilised at an experimental scale for extraction of various bioactive components such as betacyanin [260], lipase [261], bacteriocin [262] and protein [263,264], *etc*. LBF process is a simple and economical technique with high protein separation efficiency (PSE) and concentration co-efficient. It

is also cost-effective compared to other conventional techniques, owing to its recyclable nature [44].

The concept of LBF system is like the aqueous two-phase flotation (ATPF) system which relies on the salting-out effect for the separation of bioactive components. However, in this work, a novel sugaring-out method is explored. This sugaring-out method is specifically designed to counter the difficulties faced in the salting-out experiment. In the sugaring-out effect, the bioactive components are reported to preserve their properties and structure due to the lower influence of sugar solution on the target products [265]. Introduction of sugar instead of salt solution ensures that a stable pH is maintained in the system, and protects the equipment from corrosion [266].

In the sugaring-out system, the top organic phase consists of acetonitrile (ACN) instead of conventional alcohols. ACN can form a two-phase rapidly while providing a stable and friendly environment for target biomolecules. Studies have been conducted with different organic solvents and ACN has been found to be the most promising of all due to its miscibility with water at all concentrations. This feature permits ACN with the potential of extracting a wide range of biomolecules [267]. Additionally, ACN is less harmful compared to other solvents used in traditional liquid-liquid extraction systems.

However, LBF system with the sugaring-out effect is solely not efficient in extraction of protein from microalgae. This is because microalgae cells are surrounded by a rigid cell wall which preserves the compounds present in the cytoplasm and other organelles [268]. Hence, a pre-treatment is necessary to disrupt the cell wall before extraction of proteins from microalgae. It is essential to select a suitable cell disruption technique coupled with an optimum extraction method to optimise the protein recovery (PR) and avoid contamination of the target components at the downstream processes. Examples of pre-treatment of microalgae with various cell disruption techniques are pulsed electric field [269], ultra-sonication [270] and high voltage electrical discharges [271].

This study utilises electrolysis as a cell disruption method for microalgae. The electric field will be passing through the system to initiate the movement of negatively charged microalgae components to the anode, followed by rising to the top solvent phase *via* gas bubbles [44].

In the current experiment, the cell-disruption method of electrolysis was integrated with LBF system to extract proteins from *Chlorella vulgaris* FSP-E biomass. The main aim of this study is to optimise the operating parameters of the integrated LBEF system. The parameters manipulated include the microalgal biomass loading, voltage of the DC electric supply, concentration and type of sugar, concentration of ACN, air flowrate and duration of flotation system. The system was compared with the control LBF system. The scalable nature of the system was also investigated.

3.2. Experimental

3.2.1. Materials

The chemicals required for this experiment, namely, glucose, sucrose, maltose, fructose, xylose, ACN and Bradford's reagent were purchased from R&M Chemicals. Bovine serum albumin (BSA) protein required for protein assay was also purchased from R&M Chemicals. All the purchased chemicals were of analytical grade.

3.2.2. LBF equipment set-up

The apparatus for the LBF system of 500 mL was obtained from Donewell Resources of Puchong, Malaysia. The LBF set-up consists of a glass cylinder with an inner diameter of 7 cm, a height of 15 cm and a volume of 500 mL. At the bottom of the unit, a hole was drilled to connect 50 mL centrifugal tube. The air bubble filter required for flotation in the column is installed at the bottom of the cylinder. It is a sintered disk of G4 porosity with a pore size of 10μm. Compressed air was supplied through the bottom of the unit with the help of an air compressor. The flow rate of compressed air was controlled with a flowmeter (model: RMA-26-SSV, Dwyer, USA) of range between 50-200 mL/min.

3.2.3. Microalgae strain and medium selection

Chlorella vulgaris FSP-E algal strain was obtained from National Cheng Kung University of Taiwan. This specific strain was selected due to its high protein percentage (58 %) as dry matter [272]. The *Chlorella vulgaris* FSP-E microalgae were pre-cultured using BG-11 medium for 7 days followed by batch culture for 14 days. The microalgal biomass was harvested after a stagnant growth rate. The BG-11 medium consists of $NaNO_3$ as 1.5 g/L, K_2HPO_4 as 0.03 g/L, $MgSO_4.7H_2O$ as 0.075 g/L, $C_6H_8O_7$ as 0.006 g/L, Na_2CO_3 as 2 g/L, $CaCl_2.2H_2O$ as 3.6 g/L, $FeH_4N_5O_{12}$ as 0.6 g/L, EDTA as 0.1 g/L, H_3BO_3 as 2.86 g/L, $MnCl.4H_2O$ as 1.81 g/L, $ZnSO_4.7H_2O$ as 0.222 g/L, $Na_2MoO_4.2H_2O$ as 0.39 g/L, $CuSO_4.5H_2O$ as 0.079 g/L and $Co(NO_3)_2.6H_2O$ as 0.049 g/L. To prevent contamination, the initial pre-culture and the following batch-culture were carried out in the laminar flow chamber.

3.2.4. Microalgal biomass cultivation

The cultivation of microalgae was carried out in the laboratory using a small-scale photobioreactor (PBR) consisting of glass vessels with 1L working volume. The PBR was covered with artificial lights with illumination of 200 μmol/m^2/s. The temperature was fixed at around 25±1 °C. The culture medium was constantly aerated at 400 mL/min with a mixture of CO_2 and air. The mixture was agitated at 450 rpm and the cultivation was carried out for 14 days. The biomass was obtained from the broth using centrifugation at 6500 rpm for 5 min using a centrifuge (Eppendorf, 5430). The supernatant was discarded, and the remaining paste was freeze dried for 36 h. The dried biomass was stored at -20 °C.

3.2.5. Determination of microalgae concentration

The microalgae concentration determination for cell growth in the culture was carried out daily by using the optical density of the obtained sample. The culture broth was

diluted 10 times and mixed well in the cuvette. The mixture was analysed in UV-Vis Spectrophotometer (UV-1800, Shimadzu) at a wavelength of 680.8 nm.

3.2.6. LBEF integration

The LBF set-up mentioned in section 3.2.2 is integrated to equip two carbon electrodes (diameter 10mm). These electrodes are attached to an insulated polystyrene ring that rests on top of the LBF cylinder. The electrodes are placed 2 cm apart. The electric current was provided by a GW GPS-3030 single DC (0-30 V/3 A) power supply to two carbon electrodes which acted like anode and cathode, respectively. The two lead electrodes were purchased from BTSCIENCE SDN BHD, Selangor, Malaysia.

The dried microalgal biomass utilised in this experiment was first weighed to the desired amount. It was then dissolved in the prepared sugar solution of the desired concentration. The solution was mixed well and then added to the LBEF unit. This serves as the bottom phase. The ACN solution of desired concentration was added to form the top phase of the biphasic system. Compressed air of the desired flowrate was introduced to the system. The two electrodes were placed in the LBF unit, and a DC power supply of fixed voltage was provided. This initiated the experiment. The experiment was conducted for a pre-determined duration. At the end of the experiment, the two-phase solution was transferred to a measuring cylinder to note down the volume of the top and bottom phases. The experimental set-up for the experiment is shown in Figure 3.1. A 10 mL sample was collected from the top and bottom phase for analysis and the experiment was carried out in triplicate.

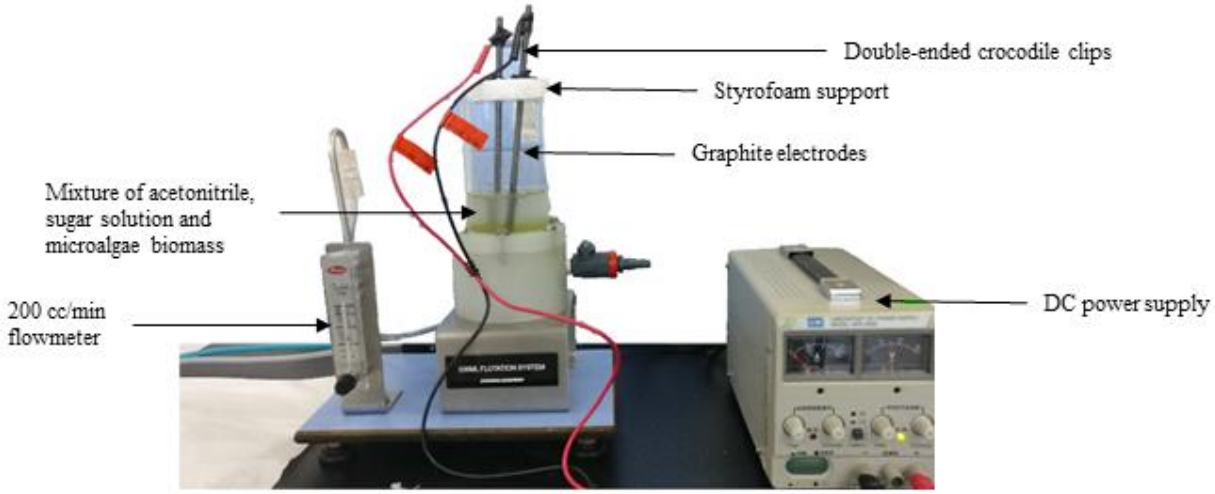


Figure 3.1 Set-up for LBEF system.

3.2.7. Protein assay

The protein content in each sample was determined by Bradford's method using BSA standard solution. The collected bottom and top phase samples of 0.2 mL were mixed with 2.5 mL of Bradford reagent. The mixture was analysed in UV-Vis Spectrophotometer (UV-1800, Shimadzu) at 595 nm wavelength after 10 min. The obtained results were compared with the standard calibration curve of BSA protein.

3.2.8. Operating parameters and optimisation of LBEF system

The protein extraction *via* LBF and electrolysis was carried out simultaneously. The method implemented here is sugaring-out instead of the conventional salting-out method. The introduction of electric current promotes cell disruption and cell contents are released effectively including the proteins. The continuous flotation will transfer these proteins from the bottom phase to the top phase by two-phase partitioning and can be extracted easily. The method employed for optimisation of protein extraction is one variable at a time (OVAT). The parameters manipulated in this study include microalgal biomass loading, voltage of DC current, position of electrodes, concentration and type of sugar, concentration of ACN, air flowrate and flotation time. The initial working

parameters were fixed at working volume of aqueous solution of 150 mL, volume of ACN at 150 mL. The biomass amount was chosen as 0.1 grams and the sugar concentration of 150 g/L. The flotation system was operated with an airflow rate of 75 mL/min for 10 min. The air was filtered using a 0.2 μm filter. The DC electric supply to the electrodes was 5 V with the tip of electrodes in the bottom phase. After manipulating all the variables, a comparison study between LBF and LBEF was conducted.

3.2.9. Determination of PSE and PR (yield)

The results were analysed using SE and PR/yield to optimise each parameter. PSE is defined as yield in terms of concentration in the top phase. It is calculated by

$$PSE = \left[1 - \frac{C_P}{C_{Pi}}\right] \times 100\ \% \quad \text{(Eq 3.1)}$$

where PSE is protein separation efficiency, C_P is protein concentration at the bottom phase at the end of the experiment, C_{Pi} is protein concentration of the initial crude microalgae.

The PR or yield is calculated by

$$PR = \frac{P_T}{P_i} \times 100\ \% \quad \text{(Eq 3.2)}$$

where PR is Protein Recovery, P_T is total protein recovered in the top phase and P_i is initial protein content in the crude microalgae feedstock.

The overall protein in the crude feedstock was determined by the following equation (3.3):

$$P_i = M_i \times Nea \times NTP \quad \text{(Eq 3.3)}$$

where M_i is microalgal biomass loading in grams, Nea is percentage of total N_2 in *Chlorella vulgaris via* elemental analysis and NTP corresponds to the total N_2 to protein conversion factor. The values for Nea and NTP were calculated as 7.81 % and 6.35 respectively [273,274].

3.2.10. Statistical analysis

The data presented was the average value of triplicates of each parameter. The values are depicted as mean±standard de*via*tion. The triplication of each parameter was conducted to validate the data obtained. Further, a One-Way ANOVA was conducted on the data obtained *via* Microsoft Excel to assess the sum of differences and confirm that $p \leqslant 0.05$.

3.3. Results and Discussion

3.3.1. Influence of microalgal biomass loading on the LBEF system

The crude microalgal biomass loading was investigated first to determine the biomass loading for determining the critical loading level of the microalgae in the LBEF system. It is noted that biomass loading potentially affects two-phase formation thus affecting the total protein yield [275]. Previous reports also suggest that crude biomass loading of greater than 5 % (w/w) results in a decrease in protein SE [276]. To evaluate the biomass loading on the system, all the other parameters were fixed at a constant and the microalgal biomass content was tested between 0.05 g to 0.5 g. This range is between the critical threshold quantities of 5 % (w/w) necessary for optimum two-phase formation. Figure 3.2 illustrates the SE and PR of varying biomass loading.

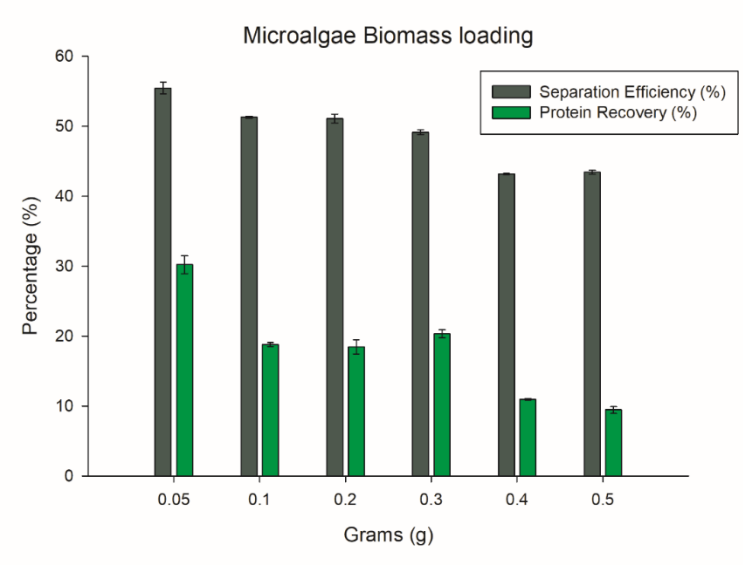


Figure 3.2 Effect of biomass loading in LBEF system with 150 mL of 100 % ACN, 150 mL of glucose solution with a concentration of 150 g/L, air supply of 75 mL/min with flotation time of 10 min and DC supply of 5 V with the tip of the electrode in the bottom phase.

From Figure 3.2, it can be noted that the SE of LBEF system steadily decreases from 0.05g to 0.3g. However, in the case of PR, there is a steep decline from 0.05 g to 0.1g. The PR remains in the same range until 0.3g biomass loading and after 0.3g there is a sharp decline in the yield. This is mainly attributed to the increase in viscosity of the bottom phase corresponding to higher biomass loading. The flotation efficiency of the system decreases due to an increase in the viscosity of the bottom phase. Therefore, yield is reduced considerably. The highest PSE and PR of 55.45±0.81 % and 0.30±1.28 % were noted for biomass loading of 0.05g, respectively. The biomass loading of 0.1g and 0.2g have similar separation efficiencies at 51.28±0.17 % and 51.07±0.62 % respectively. However, the corresponding PR is lower at 18.80±0.28 % and 18.45±1.04 % respectively. The decrease in PR is noted due to the increase in impurities and contaminants in the aqueous system at higher biomass loading [277]. In addition to that, due to the presence of higher biomass in the bottom phase, there is more precipitation on the ACN/sugar interface which hinders the efficient air supply. This results in lower

PR as the biomass loading is increased. Therefore, from this study, biomass loading of 0.05 g was selected as a constant for further variable manipulation.

3.3.2. Influence of electric current (voltage) and position of electrode on LBEF system

The DC electric current supply for this experiment was manipulated by varying voltage supply to the electrodes from 5V- 30V. The results obtained from this set-up are depicted in Figure 3.3a. As the voltage supply is increased from 5V to 15V, the PSE and PR increase. Further increase in voltage supply (20-30V) illustrates a decreasing trend in the total yield. A similar trend was observed by Leong *et al.* in the extraction of betacyanins from the peel of red-purple pitaya *via* LBEF system [260]. According to Leong *et al.*, the mass transfer in the experiment may have reached an equilibrium stage and additional of voltage supply is unnecessary for the system [260]. The highest PSE and PR of 55.16±1.25 % and 43.21±1.58 % were obtained at a DC supply of 15V, respectively. Although the PSE at 5V (55.45±0.81 %) is like that of 15V, the corresponding PR is low (30.21±1.27 %).

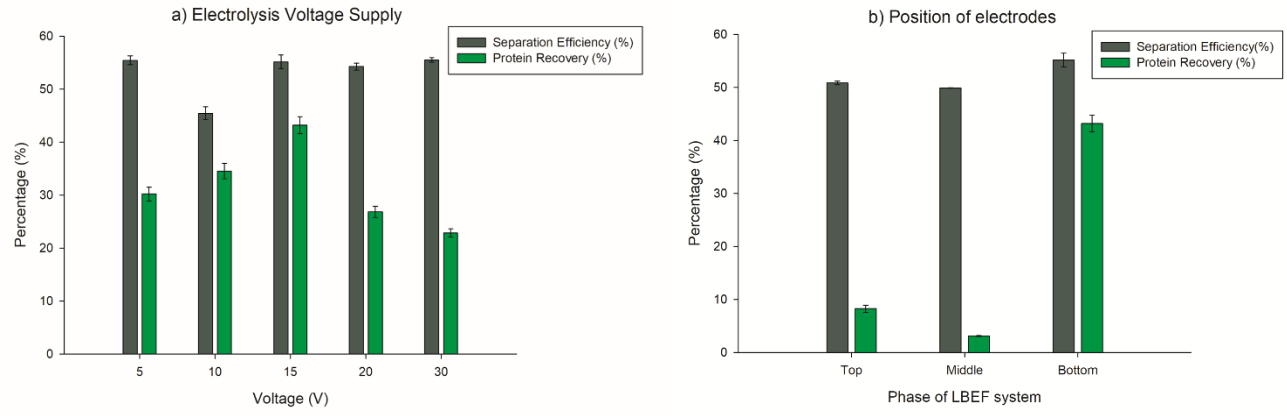


Figure 3.3 Effect of (a) voltage supply (V) and (b) electrode position in LBEF system.

The supply of electricity in the LBEF system causes electropermeabilisation of the microalgae cell membrane. This enhances the extraction of proteins from microalgal biomass. An increase in the permeability of cell membrane certainly increases the

amount of proteins released from the cell structure to the external solution [278]. The application of voltage or electric field to the system is a non-thermal process. The electric field ranging from 5V-30V passes through the cell structure and initiates membrane rupture as the cell membrane is dipole in nature [279]. The mass transfer energy of the system increases due to the addition of electric field. When the electric voltage is supplied to the system, the ions in the liquids move from anode or cathode depending on the charge of the cells. The microalgae cell carries a negative charge on the cell membrane and thus move towards anode when the electric field is introduced. This produces O_2 and H_2 gas at the electrodes. The proteins bind to these bubbles and rise to the top ACN-rich phase. Emission of H_2 and O_2 bubbles results in higher turbulence in the two-phase system. Statistical analysis of this study suggested that the data obtained is significant ($p<0.05$) and 15V of DC electric field supply is enough to induce higher SE and PR. Therefore, 15V of DC supply will be utilised in the next parameters.

After operating the LBEF system at 15V, the position of the tip of the electrodes was analysed. The tip of the electrode was positioned at the bottom, top and middle phases. The results for this set-up are depicted in Figure 3.3b. The highest SE and PR were observed when the tip of the electrode was at the bottom phase. When the tip of the electrode was at the bottom phase, the PSE was 55.16±1.25 % and PR was 43.21±1.58 %. The lowest PSE and PR of 49.88±0.04 % and 3.11±0.08 % were observed when the tip was placed at the middle or the interface of the two-phase system, respectively.

As observed from Figure 3.3b, it is evident that electric field supply to the bottom phase results in higher protein extraction efficiency. As the microalgal biomass is present in the bottom phase, the supply of electric field to the bottom phase releases the protein and the subsequent flotation carries the proteins to the top phase. However, when the electric field is supplied to the middle and top phase, the protein extraction efficiency is reduced

due to the absence of microalgal biomass initially. As the flotation progresses and proteins are extracted in the top phase, the electric field is effective. However, it cannot compete with the electrode tip in the bottom phase. Therefore, in further optimisation experiments, the tip of the electrodes is immersed in the bottom phase.

3.3.3. Effect of sugar concentration and type of sugar on LBEF study

In the conventional salting-out two-phase systems, the type of salt and its corresponding concentration plays a major role. These two parameters are vital for effective two-phase formation and thus affect the efficiency of the system. Similarly, in the sugaring-out effect, optimisation of the type and concentration of sugar is necessary. In this section, the sugar concentration for glucose is optimised. The ACN molecules interact with water molecules *via* hydrogen bonding and dipole-dipole interactions to form three-dimensional clusters (N-H-O complexes) [280]. The formation of two-phase in the sugaring-out system is predicted due to the substitution of hydrogen bonds between ACN-water by the added sugar molecules [265]. Therefore, ACN molecules are separated and form a two-phase system. According to Wang *et al.*, an increase in sugar concentration should enhance and strengthen the two-phase formation [281]. Higher sugar concentration also ensures higher ACN volume in the top phase for recycling [281]. Figure 3.4a illustrates the results of this experiment with glucose concentration ranging from 100 g/L to 300 g/L.

As previously suggested by the literature study, the results of the experiment follow a similar trend. As the glucose concentration is increased from 100 g/L to 300 g/L, there is a steady increase in SE. The PSE rose from 50.01±0.08 % at 100g/L to 69.60±1.52 % at 300 g/L. The PR for glucose concentration of 100 g/L is very low (3.36±0.16 %) due to the weak two-phase formation. At 100 g/L, there are fewer sugar molecules to replace the ACN-water complex. This results in weaker two-phase formation and introduction of turbulence due to flotation eventually collapse the two-phase formed. However, there is

a steady increase in PR from 150 g/L (43.20±1.58 %) to 300 g/L (59.46±2.03 %). As the sugar concentration of the bottom phase increases, there are lesser hydrogen bonds available for the released proteins to attach, they rise to the top phase and can be extracted. An increase in the volume of ACN with higher glucose concentration also enhances extraction of non-polar proteins as ACN is hydrophobic (non-polar) compared to water [282]. Statistical analysis of this study suggested that the data obtained is significant ($p<0.05$) and 300 g/L of sugar concentration was selected for optimisation of further parameters.

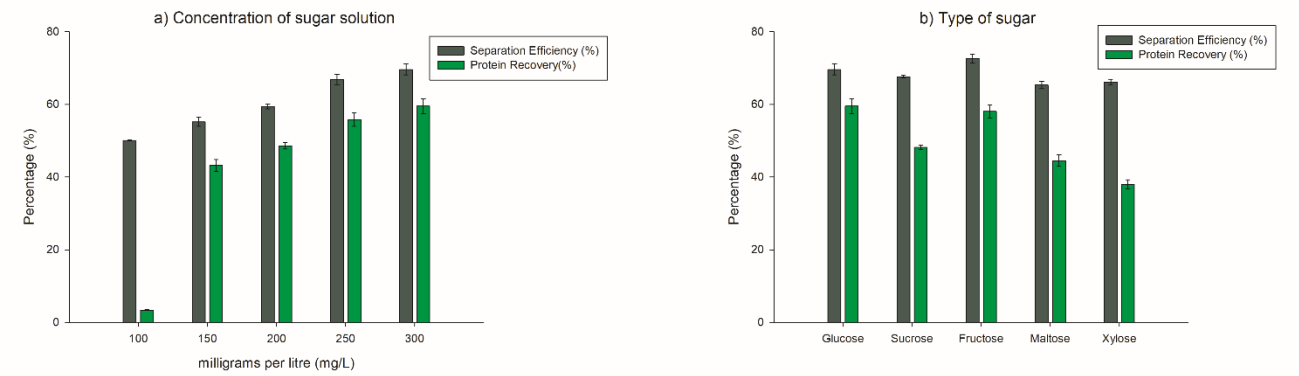


Figure 3.4 Effect of (a) glucose concentration (g/L) and (b) tPRe of sugar on LBEF system.

As the concentration of sugar was determined, the corresponding type of sugar was studied. For this study, five different types of sugars were selected, namely glucose, sucrose, fructose, maltose and xylose. Their PSE and PR are illustrated in Figure 3.4b. Based on the results from Figure 3.4b, it is evident that sucrose, maltose and xylose showed the lowest PR of 48.17±0.58 %, 44.48±1.61 % and 37.92±1.33 % respectively. The highest PR was reported by glucose at 59.46±2.03 %, followed by fructose at 58.02±1.84 %. The PSE was highest for fructose at 72.62±1.20 % followed by 69.59±1.52 % for glucose. The lowest PSE was observed by maltose followed by xylose and sucrose at 65.29±1.01 %, 66.14±0.72 % and 67.61±0.36 % respectively.

Previous reports suggest that two-phase formation is largely influenced by the placement of hydroxyl group on the 4th carbon of the circular ring of the sugars. For monosaccharide sugars like glucose, fructose and xylose, glucose has higher PR compared to fructose and xylose. Fructose is a ketose with 5 chain carbon ring and xylose is an aldose on a 5 chain carbon ring whereas glucose has 6 chain ring which increases the availability of the number of hydrogen bonds [283]. The low PR for sucrose and maltose can be explained by their molecular structure. Both these sugars are disaccharides. Sucrose contains one glucose and one fructose molecule compared to two glucose molecules of maltose. They each can form eight hydrogen bonds whereas glucose and xylose can form only five and four bonds respectively [282]. However, the difference in the number of moles of sucrose (0.87 moles) and maltose (0.87 moles) for the same mass of glucose (1.66 moles) at 300 g/L limits the efficiency of two-phase formation [283]. Statistical analysis of this study *via* one-way ANOVA indicated that the data obtained is significant ($p<0.05$) and hence, glucose was selected for further optimisation.

3.3.4. Effect of concentration of ACN on LBEF system

In this section, the effect of concentration of ACN on the LBEF system was explored. The ACN concentration was manipulated from 70-100 %. Figure 3.5 illustrates the results obtained from various concentrations of ACN. The ACN was diluted to 70 %, 80 %, 90 % and 100 %. The experiments was conducted with the corresponding concentration as the top phase of the LBEF system. According to Figure 3.5, it is evident that ACN concentration of 100 % exhibits the highest PSE and PR of 69.59±1.52 % and 59.45±2.03 %, respectively. As the concentration of ACN was reduced, the protein SE and PR observed a decreasing trend. The lowest PSE (49.99±0.09 %) and PR (3.33±0.17 %) were obtained at 70 % ACN concentration. At such a low concentration, the two-phase formation was weak and could not sustain it throughout the experiment. As the concentration of ACN is reduced, the ACN and water molecules bind firmly due to

the hydrogen bonds between them. As water molecules are higher in number at such low ACN concentration, the glucose will form hydrogen bonds with these water molecules and result in weaker two-phase formation [264]. Therefore, in further optimisation, ACN with 100 % concentration was employed.

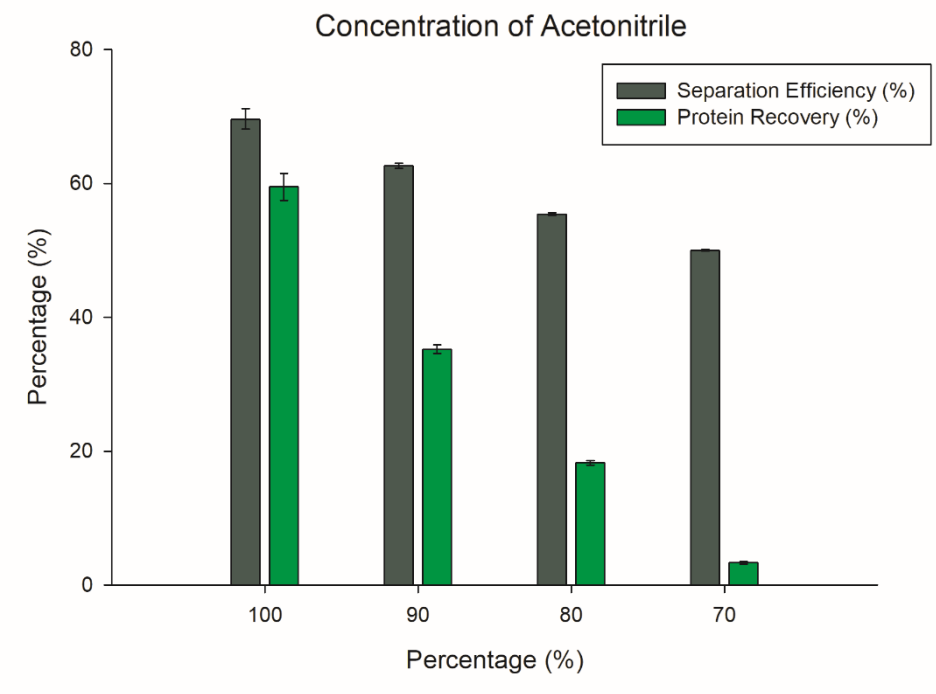


Figure 3.5 Effect of ACN concentration (%) on LBEF system.

3.3.5. Effect of air flowrate and flotation time on the LBEF system

The air flowrate for LBEF experiment plays an important role in optimising the PSE and PR of protein from microalgae. In this part of the experiment, the air-flowrate for the flotation system was manipulated from 50 mL/min to 200 mL/min. The results are shown in Figure 3.6a. The PSE for 50, 75, 100, 125 and 200 mL/min of air flowrate were 70.18±1.26 %, 69.59±1.52 %, 68.49±1.33 %, 67.84±0.90 % and 68.14±0.88 % respectively. All these values range from 65-70 %, however, at an air flowrate of 150 mL/min, the PSE is the highest at 72.81±0.57 %. In the case of PR, a similar trend is followed for air flowrates of 50, 75, 100, 125 and 200 mL/min with values ranging from 57-60 %. Although, at 150 mL/min, the PR is highest at 66.46±0.71 %.

The increase in air flowrate from 50 mL/min to 150 mL/min illustrates an escalation in PR. However, with an increase in the air flowrate, the PR decreases. As the air flowrate increases, more air bubbles are introduced to the bottom phase. This increases the efficiency of bioactive protein molecules to rise above the top phase. The turbulence caused by increasing air flowrate also ensures higher protein adsorption on the air bubbles and thus higher PR [284]. However, after crossing 150 mL/min, the PR decreases from 66.46±0.71 % to 57.52±1.17 %. This steep decrease in PR can be explained by the increase in turbulence caused by a higher air flowrate. At 200 mL/min, the time of contact between the protein and air bubbles is very low and thus inefficient adsorption is observed. The intense turbulence due to high air flowrate is also responsible for re-dissolution of the bioactive proteins in the sugar solution [285]. This does not promote mass transfer and thus reduces the PR of the whole system. Statistical analysis of this study suggested that the data obtained is significant ($p<0.05$) and hence, 150 mL/min of air flowrate was utilised for further optimisation of this experiment.

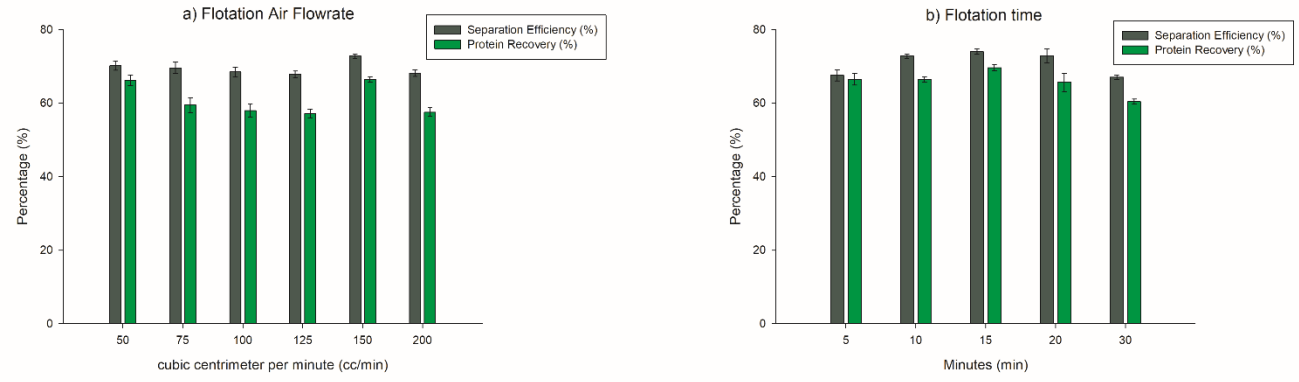


Figure 3.6 Effect of (a) air flowrate (mL/min) and (b) flotation time on LBEF system.

The flotation time for the chosen air flowrate was examined in this part of the experiment. The flotation time tested was 5, 10, 15, 20 and 30 min, respectively. It is evident from Figure 3.6b that protein SE and PR increase progressively with an increase in flotation duration. However, after 15 min, prolonged flotation reduces the PSE and PR,

respectively. The PSE for 5, 10, 15, 20 and 30 min were 67.58±1.52 %, 72.81±0.57 %, 73.99±0.74 %, 72.87±1.97 % and 67.01±0.55 % respectively. The corresponding PR values were 66.49±1.57 %, 66.46±0.71 %, 69.66±0.86 %, 65.63±2.49 % and 60.40±0.66 % respectively. The SE and PR increase linearly with an increase in flotation time. This phenomenon is observed due to a higher contact duration between proteins and air bubbles introduced in the system [44]. Although, as the flotation time is prolonged, an opposite trend is observed. This might be because the system is in equilibrium at 15 min and an increase in flotation duration does not provide higher efficiency. Statistical analysis of this study suggested that the data obtained is significant ($p<0.05$). Therefore, a flotation duration of 15 min was selected as the last step for optimisation of this experiment.

3.3.6. Comparison between LBEF and LBF and scale-up approach

As all the parameters were optimised, a comparison study was conducted between the newly optimised LBEF system with the control LBF set-up. This was mainly conducted to investigate the change in PR and PSE induced by the electrolysis pre-treatment of the microalgae cells. The results are illustrated in Table 3.1. In the LBF set-up, the electric voltage supply was not provided. In this case, there was no external cell disruption technique involved and thus resulted in lower SE and PR. The lack of external electrolysis effect by the electric field in the microalgae rich bottom phase limits the cell wall disruption, therefore the amount of protein released from microalgae cells is low, which is reflected in Table 3.1.

Table 3.1 A comparison study of LBF and LBEF system in a large-scale approach.

	Protein separation efficiency (%)	**Protein recovery (%)**
	Type of system	
LBF	61.58±0.36 %	48.78±0.48 %
LBEF	73.99±0.74 %	69.66±0.86 %

Integrated LBEF system		
Small-scale	73.99±0.74 %	69.66±0.86 %
Large-scale	52.87±1.23 %	73.29±0.70 %

The integrated LBEF system optimised in this experiment was conducted at a larger scale. The initial experiment was conducted with a working volume of 300 mL (150 mL of sugar solution and 150 mL of ACN). In the large-scale set-up, the experiment was conducted at 5 times the initial volume. The results are demonstrated in Table 3.1. SE was reduced; however, the PR was a bit higher than the small-scale experiment. Therefore, this experiment can be verified with positive results from a large-scale system.

3.4. Conclusions

This study investigates the effects of various factors for protein extraction using an integrated method for increasing the overall efficiency of the LBF system. The results suggest that the SE and PR of the LBEF system increase significantly when compared with the simple LBF system. Moreover, utilising the sugaring-out effect eliminates the corrosion and equipment damage caused by conventional salts in the biphasic system. The result after optimisation of all the parameters was obtained with 0.05g of microalgal biomass with 15V of DC current supply with the tip of the electrode at the bottom phase. The sugar concentration of glucose was 300 g/L and the ACN concentration was 100 %. The air flowrate of the flotation system was 150 mL/min with a flotation time of 15 min resulted in optimum values for PSE and PR. The optimised SE of the LBEF system was 73.99±0.74 % and PR of 69.66±0.86 % compared to LBF, the SE was 61.58±0.36 % and PR was 48.78±0.48 %. The optimised parameters were utilised in a 5× time scaled-up LBEF system. The SE and PR were noted at 52.87±1.23 % and 73.29±0.70 % respectively. The major drawback of this study was the smaller magnitude of the voltage supply and utilisation of high voltage pulsed electricity is necessary to effectively study the cell disruption of microalgae. Furthermore, future studies can be conducted with different optimisation approach that will take into consideration the interaction between

the selected parameters. This novel, simple method is fast and reliable and can be a breakthrough in solving the tailbacks of conventional extraction technologies.

CHAPTER 4

4. Biorefinery of *Chlorella sorokiniana* using U-LTF system

4.1. Introduction

Apart from a few developed countries, developing and least developed countries are striving towards urbanization. This has resulted in over-exploitation of fossil fuels, depletion of Earth's non-renewable resources, emission of harmful greenhouse gases which caused catastrophic anomalies in the environments. These effects are evident in recent decades with increasing reports of deaths due to extreme heatwaves, unexpected floods, water-borne and air-borne diseases [286,287]. This linear model of resource utilisation is unsustainable and thus research has been diverted to accommodate a much greener circular model for an ecologically balanced approach [288]. Many developed countries such as USA, UK, and Japan have taken the first step towards achieving this goal. The four major challenges for the 21st century are poverty, hunger, global warming and pollution (air, water and land) [289]. In this context, biorefinery of renewable feedstock/resources, as opposed to conventional petroleum-based refinery, is of much interest [290] to harness multiple products from a single source [291].

Microalgae are unicellular autotrophic photosynthetic organisms with the potential of reducing global warming and water pollution while providing high value-added products [292]. Microalgae cells uptake the nutrients in wastewater or seawater and grow rapidly in the presence of sunlight and anthropogenic CO_2 [293]. Microalgae secrete high value-added products such as lipids, proteins, carbohydrates, polyunsaturated fatty acids, antioxidants and vitamins [40]. Despite its rich nutrients content, commercial production of microalgae is challenged by the high cost associated with harvesting, extraction and purification of the targeted compounds [294,295]. The primary metabolites and bioactive components in microalgae are enclosed in a rigid cell wall. Disruption of the cell wall involves high costs and complicated downstream processes. Current techniques

employed in the extraction of targeted compounds from microalgae include bead beating, high-pressure homogenization, ultrasound, freeze-thawing, autoclaving and supercritical CO_2 [79]. A conventional microalgae extraction plant incorporates centrifugation and precipitation. The target compound is usually separated by chromatographic methods and purified *via* lyophilisation. Selection of processes that involve numerous operational units, high energy expenditure and complex process control thus resulting in the utilisation of only high-value niche compounds [296].

To overcome these limitations, simple phase separation processes such as liquid biphasic system (LBS) and three-phase partitioning (TPP) were introduced in the extraction of metabolites from microalgae. A combination of LBS and flotation has successfully extract various bioactive components such as lipase, proteins, bacteriocin, betacyanin, *etc.* [260–263]. Flotation-assisted LBS, also known as LBF system, involves addition of two immiscible liquids with a specific concentration to form a stable interface. The bioactive components are adsorbed to the air bubbles introduced in this system and ascended to the top organic layer *via* SS. LBF system is advantageous owing to its high concentration co-efficient, high extraction efficiency, economic feasibility, continuous operation and ease of handling. Higher concentration co-efficient corresponds to higher protein concentration in the alcohol-rich top phase [44,297]. However, LBF system is limited to extraction of a single component from the biomass feedstock [298]. TPP is a simple process based on the separation of surface-active components in three phases. These phases consist of the bottom salt phase, middle interface protein precipitate and top organic phase. In TPP system, tert-Butyl alcohol (t-butanol) is selected for the upper organic layer due to its high boiling point and low flammability. The large molecular weight of t-butanol (74.12 $g \cdot mol^{-1}$) prevents denaturation of proteins in the interfacial layer. Previous research conducted *via* TPP of microalgal biomass successfully extracted proteins and lipids in the interface middle phase and upper organic layer, respectively

[299]. However, the stirring operation unit required for phase separation in TPP systems is unfavourable. To overcome this obstacle, SS technique utilised in LBF was introduced in this study to TPP. The resulting liquid triphasic flotation (LTF) technique with a shorter process time was introduced.

In the current study, LTF system is incorporated with ultrasonication to extract lipids and proteins *via* a biorefinery approach, as shown in Figure 4.1. A comparison study of conventional TPP system and control (with the absence of cell-disruption technique) was investigated. Various parameters related to LTF system such as microalgal biomass loading, concentration of salt, volume ratio of t-butanol:ammonium sulphate ($(NH_4)_2SO_4$) solution, ultrasonication interval, air flowrate and flotation time were investigated. An additional upper organic phase recycling study was also conducted to confirm the circular nature of LTF system.

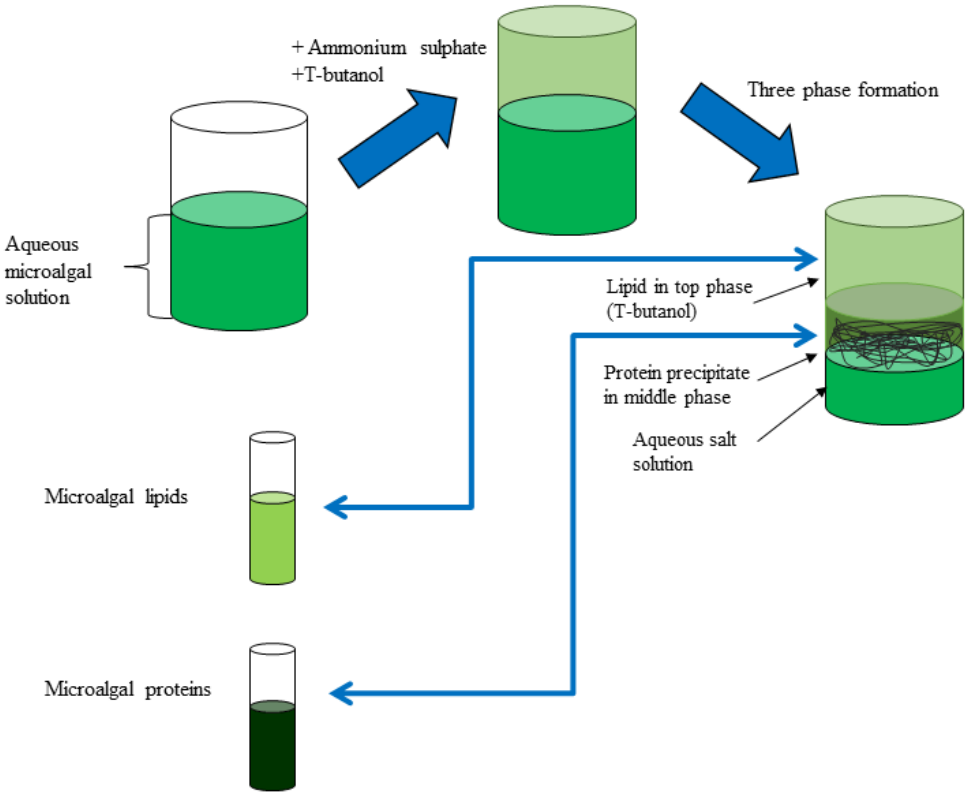


Figure 4.1 Biorefinery approach of *Chlorella sorokiniana* by LTF system.

4.2. Experimental

4.2.1. Materials

T-butanol, $(NH_4)_2SO_4$, tris-HCl buffer, sodium hydroxide (NaOH), Bradford's reagent, tri-ethanolamine (TEA), copper (II) nitrate ($Cu(NO_3)_2$), methanol and chloroform were purchased from R&M Chemicals. All the purchased solvents and chemicals were of analytical grade and used without further purification.

4.2.2. LTF equipment set-up

The LTF apparatus was obtained from Donewell Resources (Puchong, Malaysia). The equipment set-up is following a study reported by our group [146]. Briefly, the apparatus was made of a glass cylinder with an inner diameter and height of 7 and 15 cm, respectively. The total volume of the cylinder was 500 mL. A centrifugal tube was connected at the bottom of the equipment for air supply. The bottom of the unit consisted of a bubble filter made up of sintered disk with G4 porosity and pore size of 10 µm. An air compressor was utilised for the supply of compressed air to the bottom of this set-up. The compressed air flow rate was controlled by a flowmeter (RMA-26-SSV, Dwyer, USA) with a range of 50-200 mL/min.

4.2.3. Microalgae cultivation

The microalgae strain of *Chlorella sorokiniana* CY-1 was obtained from National Cheng Kung University of Taiwan. The selected species composed of 40 % protein, 18-22 % lipids and 30-38 % carbohydrates [300]. The microalgae were pre-cultured for 7 d, followed by main batch culture of 14 d in an enclosed laboratory-scale PBR. The medium used for microalgae culture was BG-11. The microalgae culture was provided with constant illumination of 200 µmol/m^2/s, continuous aeration at 400 mL/min and agitation of 450 rpm. After 14 d, the microalgae culture was harvested by centrifugation at 4960 g for 5 min, followed by freeze drying for 24 h. It was stored at -20 °C and experimental runs were conducted within 2 weeks.

4.2.4. Determination of microalgae cell concentration

The microalgae cell concentration in the culture broth was carried out daily by determining the optical density of the sample. The culture broth was initially diluted 10 times before characterised by UV-Vis spectrophotometer (UV-1800, Shimadzu) at a wavelength of 680.8 nm.

4.2.5. Operation of U-LTF system

The bottom of LTF system was connected to a compressed air pump. To prevent backflow of solvents in the connecting tube, sufficient air was provided before adding the bottom aqueous phase. The bottom aqueous phase of LTF system consisted of microalgal biomass dissolved in 100 mL of $(NH_4)_2SO_4$ salt solution in pre-determined concentration. The alcohol phase consisting of 100 mL of t-butanol was added to the aqueous bottom phase to form a three-phase system. The air bubbles of flowrate between 20-25 mL/min were supplied for 5 min. After the system was settled, desired air flowrate was provided for the selected time duration. During the operation of LTF system, an ultrasonication probe (Bandelin Sonopuls UW 2200, Germany) was submerged in the system for simultaneous cell disruption. The sonicator probe with titanium horn sonotrode (TT 13/FZ) was set at 20 % amplitude with a pulse mode of 5s ON/ 20s OFF at room temperature. At the end of each run, the three phases were collected *via* a tap located at the bottom of the equipment. The respective volume of each phase was noted. All the subsequent analyses were conducted in triplicates.

4.2.6. Optimisation of operating parameters of LTF system

The optimisation of parameters manipulated in this experiment was conducted by OVAT method. The parameters manipulated are listed in Table 4.1. The selection of parameters was based on SE, proteins recovery and lipids yield. All the experimental runs were performed at room temperature. The top, middle and bottom phases were collected for protein analysis while the top phase was collected for lipids analysis.

Table 4.1 Initial settings for manipulated parameters of TLF system.

Manipulated parameters	Variables	Unit	Initial parameter
Microalgal biomass loading	0.25, 0.5, 0.75, 1.0	w/v%	0.5
Concentration of salt	0.25, 0.3, 0.35, 0.4, 0.5	w/v%	0.3
Volume ratio	1:0.5, 1:1, 1:1.5, 1:2	salt solution:t-butanol	1:1
Ultrasonication duty	5/10, 5/20, 10/20, 20/20	NA	20/20
Flotation air flowrate	75, 100, 125, 150	mL/min	100
Flotation time	5, 10, 15, 20	min	10

4.2.7. Protein assay

The protein concentration after extraction from microalgae was analysed by Bradford's method. 2 mL of Bradford reagent was added to 0.2 mL of sample. The mixture was measured spectro-photometrically at a wavelength of 595 nm after 10 min. The protein concentration was determined by comparing the results from the standard calibration curve of BSA at 595 nm. The BSA concentration in the calibration curve was in a range of 0-1.2 g/L.

4.2.8. Lipid assay

The lipid assay was conducted on the alcohol-rich top phase. T-butanol was evaporated in a rotary evaporator (EYELA) and the residual lipid solids were collected. The lipid analysis of the extracted sample was conducted by Cu-TEA-FA complex formation as described by [301]. The residual lipids were saponified and observed spectrophotometrically at a wavelength of 260 nm after complex formation with $Cu(NO_3)_2$. The obtained results were compared with the standard calibration curve obtained with sodium palmitate. The sodium palmitate standard range was between 0-0.15 mg/mL. The analysis of samples was carried out in triplicates.

4.2.9. Determination of protein SE, PR and LR

The protein analysis was conducted by calculating the SE and recovery of total proteins to optimise each parameter. PSE is defined as yield in terms of concentration in the top phase. It is calculated by equation (4.1).

$$PSE\ \% = \left[\frac{C_m V_m}{C_t V_t + C_m V_m + C_b V_b}\right] \times 100\ \% \quad \text{(Eq 4.1)}$$

where PSE is protein separation efficiency, C is protein concentration and V is volume of respective phases. The subscript represents the top, middle and bottom phase.

The PR is calculated by equation (4.2).

$$PR = \frac{P_m}{P_i} \times 100\ \% \quad \text{(Eq 4.2)}$$

where PR is protein recovery, P_m is total protein recovered in the middle phase and P_i is initial protein content in the crude microalgae feedstock.

The overall protein in the crude feedstock was determined by the following equation (4.3).

$$P_i = M_i \times Nea \times NTP \quad \text{(Eq 4.3)}$$

where, P_i is overall protein in crude extract, M_i is microalgal biomass loading in grams, Nea is percentage of total N_2 in *Chlorella sorokiniana via* elemental analysis and NTP corresponds to the total N_2-to-Protein conversion factor. The values for Nea were calculated *via* elemental analysis and was reported to be 4.14 %. The NTP ratio is a constant with corresponding value of 4.78 [273,274].

Lipids extraction is analysed with respect to lipids equivalent to palmitic acid (C18:0). The lipids yield is calculated by equation (4.4).

$$LR = \frac{L_T}{L_i} \quad \text{(Eq 4.4)}$$

where, *LR* is lipid yield, L_T is lipids content of the extracted sample from alcohol-rich top phase and L_i is lipids content of initial microalgae feedstock with the respective biomass concentration.

4.2.10. TPP comparison study

The TPP comparison study was conducted by optimised U-LTF parameters. For this study, microalgae solution of pre-determined concentration was mixed with selected salt, followed by the addition of t-butanol. This solution was stirred at 200 rpm for 1 hr. The study was conducted at room temperature. After stirring the TPP for 1 hr, it was settled for 1 hr. The three phases were collected, and their respective volume was noted. The middle protein precipitate was dissolved in 0.1M Tris-HCl buffer and further analysed. The top phase was analysed for both protein and lipid content. The experiment was conducted in triplicates.

4.2.11. Recycling study

The recyclability of U-LTF system was conducted by recovering the top alcohol phase and utilising it for the next extraction cycle. The top alcohol phase was collected and evaporated in the rotary evaporator (EYELA) to obtain t-butanol. T-butanol was analysed for purity with a digital refractometer (ATAGO). The alcohol purity in Brix No. (0.0-93.6 %) was obtained and compared with calibration data of t-butanol. The recycled alcohol was used for the second extraction cycle and the corresponding PR and LR was calculated. This recycling and extraction cycles were repeated.

4.2.12. Statistical analysis

The PR, protein SE and lipids yield were analysed by One-Way ANOVA with a two-tailed t-test. Each run in this experiment was triplicated and the data is mentioned in this paper as mean±standard deviation. The statistical analysis of the data was conducted by Microsoft Excel to assess the sum of differences with significance $p \leqslant 0.05$.

4.3. Results and Discussion

4.3.1. Effect of biomass loading and salt concentration on LTF system

The effect of biomass loading on U-LTF system was investigated with the initial parameters described in Table 4.1. The biomass loading in the range of 0.25-1.0 w/v% was investigated in this sub-section. The SE and recovery of proteins is depicted in Figure

4.2a. Figure 4.2a also indicates the lipid yield equivalent to palmitic acid (C18:0). The highest PR of 96.50±3.16 % and LR of 15.27±0.14 % was observed at biomass loading of 0.5 w/v%. Although, the SE of protein at biomass loading of 0.5 w/v% was comparable to other settings at 89.98±7.96 %. It is evident from Figure 4.2a that microalgal biomass concentration of 0.5 w/v% is the most ideal setting. Based on Figure 4.2a, an increase in biomass loading results in low PR and SE as well as low LR. This was observed due to an increase in viscosity of LTF system with higher biomass concentration, which hinders the performance of the flotation system [259]. As the biomass concentration increases, various impurities and other biomolecules from *Chlorella sorokiniana* accumulate in the top and intermediate phases. Thus resulting in a thicker middle phase and lowering the SS efficiency [277]. This subsequently reduces the lipid recovery of LTF system. The statistical analysis conducted on the SE, PR and LR obtained from biomass loading significantly differed with p-value <0.05. Therefore, 0.5 w/v% of biomass loading was selected for further studies.

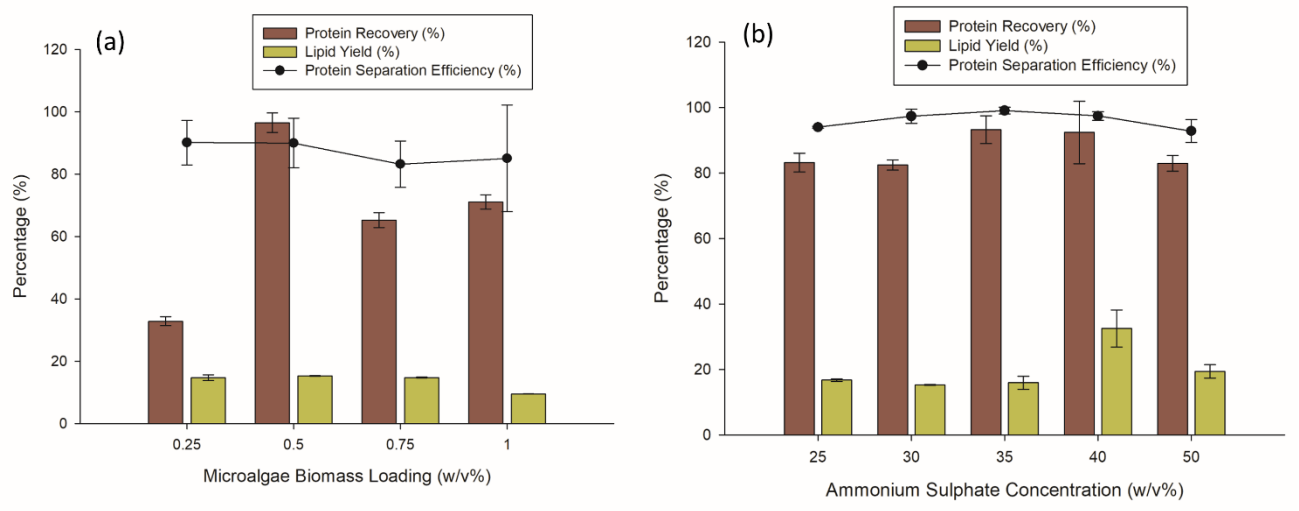


Figure 4.2 PR, PSE and LR with respect to a) microalgal biomass loading and b) concentration of salt solution on U-LTF system.

In addition to microalgal biomass content, the concentration of $(NH_4)_2SO_4$ salt plays an important role in LTF system. In LTF system, $(NH_4)_2SO_4$ and t-butanol form a kosmotrophic system due to a synergistic effect between them [302]. The results obtained from this study are depicted in Figure 4.2b. The highest PR of 93.25±4.27 % was observed at $(NH_4)_2SO_4$ concentration of 35 %. However, it was comparable to the PR of 92.41±9.53 % at 40 % $(NH_4)_2SO_4$ solution. Their protein SE followed the same trend, *i.e.*, 99.05±1.05 % at 35 w/v% and 97.42±1.32 % at w/v%. However, the lipid yield at 40 % ammonium solution was very high (32.55±5.66 %) compared to that of 35 w/v% (15.95±1.96 %). Beyond 40 % of salt solution, there was a decrease in PR as well as LR. This can be attributed to the denaturation of proteins and structural changes to lipids due to pH change at high salt concentration. The lipid yield at low salt concentration can be explained by the lower ionic strength of the LTF system, which results in poor intramolecular and intermolecular electrostatic forces between the three phases [303]. The statistical analysis conducted on the SE, PR and LR obtained from this study significantly differed with p-value <0.05. Therefore, 40 w/v% of $(NH_4)_2SO_4$ concentration was selected for further studies.

4.3.2. Effect of volume ratio of t-butanol:salt solution and ultrasonication duty

For this section, the volume ratio of salt solution:t-butanol was determined. From the previous study, it is evident that the concentration of salt and its respective behaviour with organic phase affects the performance of LTF system. Thus, in this study, various volume ratio combinations were tested to determine the optimum parameter. The volume ratios of 1:0.5, 1:1, 1:1.5 and 1:2 corresponding to salt solution:t-butanol were investigated. This parameter is crucial in determining the synergistic of the salt solution and t-butanol incorporated in LTF system. The results from this study are depicted in Figure 4.3a. In the figure, it is noticeable that the PR of volume ratio of 1:0.5, 1:1 and

1:1.5 are comparable at 90.45±2.87 %, 91.99±4.1 8 % and 92.26±1.90 %, respectively. However, with the volume ratio of 1:2, the PR and SE decreased to 85.28±6.07 % and 95.80±1.74 %, respectively. This is mainly attributed to the denaturation of proteins with a high volume of t-butanol [299,304]. For lipid yield, a slightly different trend was observed. The lipid yields of volume ratio 1:1, 1:1.5 and 1:2 was similar at 32.55±5.66 %, 32.74±0.36 % and 29.48±0.71 %. Panadare *et al.* and Qui *et al.* also observed a similar trend of high lipid yields at the volume ratios of 1:1 and 1:1.5. It is attributed to improved solubility of lipids with a high volume of t-butanol [305,306]. The volume ratio of 1:0.5 resulted in the least lipid yield of 16.71±0.21 %. Thus, the volume ratio of 1:1.5 with respect to salt solution and t-butanol enhanced the salting-out nature of LTF system by withdrawing water from the salt solution [307]. The statistical analysis of the results obtained from this study suggested that the data differed significantly with p-value <0.05. Therefore, the volume ratio of 1:1.5 was selected for further experiment.

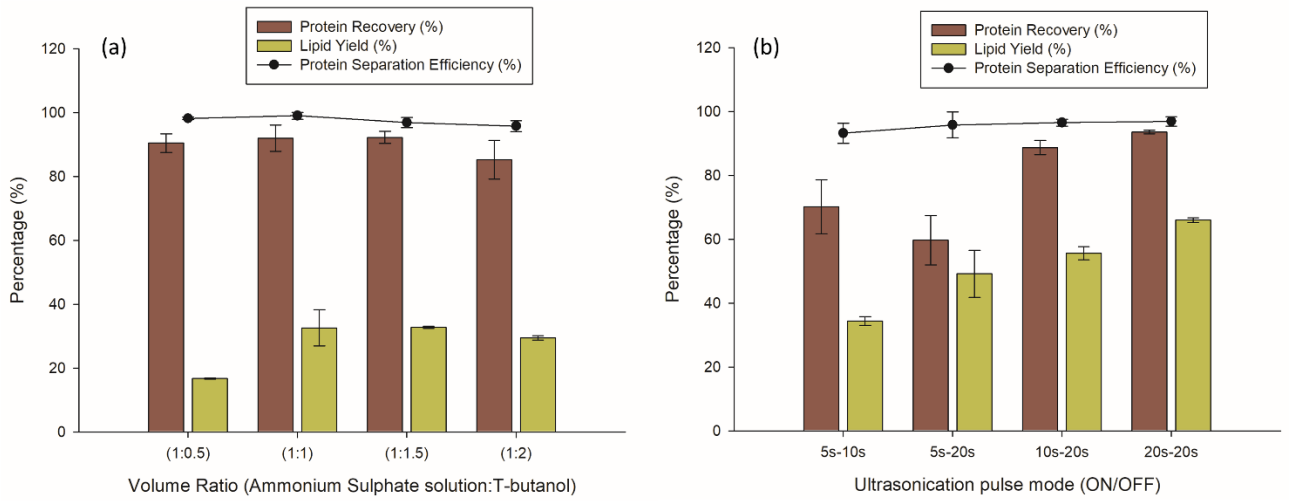


Figure 4.3 PR, PSE and LR with respect to a) volume ratio and b) ultrasonication pulse duty on U-LTF system.

The ultrasonication settings were manipulated with the selected volume ratio to optimise the LTF system. The results of this study are depicted in Figure 4.3b. The ultrasonication

pulse mode was only conducted for 5 min. The range of modes for ultrasonication was selected from previous studies conducted [264,308]. The time limit of 5 min was chosen because with prolonged ultrasonication, there is an energy transformation from sound to heat. This leads to an increase in temperature of LTF system thus resulting in the denaturation of proteins [309]. Moreover, Gerde *et al.* indicated that prolonged ultrasonication (300-3600 s) results in the production of free radicals due to the breakdown of water molecules. This affects the quality of oil produced from the extracted lipids [310]. Therefore, for this study, the ultrasonication pulse mode range chosen was 5s/10s, 5s/20s, 10s/20s, 20s/20s (ON/OFF) for a total duration of 5 min with an amplitude of 20 %. From Figure 4.3b, at pulse setting of 20s ON/20s OFF, the highest PR and SE of 93.57±0.55 % and 96.93±1.49 %, respectively, were observed. The SE of all the settings were comparably ranging from 93.19 % to 96.93 %. The PR, however, decreased for pulse settings of 5s ON/ 10s OFF. This is probably due to a higher resting period compared to 5s ON/ 10s OFF setting. However, there was a steady increase in lipid yield in every setting. The highest lipid yield was observed for 20s ON/ 20s OFF setting at 65.99±0.75 %. The statistical analysis of this parameter revealed that the data differs significantly which is indicated by p-value <0.05. Therefore, ultrasonication pulse mode of 20s ON/ 20s OFF for 5 min was selected for further experiments.

4.3.3. Effect of air flow rate and flotation time

This study investigated the effect of manipulating the SS conditions on the LTF system. The two important parameters governing SS were air flow rate and flotation time. For studying the effect of air flow rate, the flowmeter readings in the range of 75 mL/min to 150 mL/min were selected based on previously conducted studies. The results from this study are depicted in Figure 4.4a. According to Figure 4.4a, the highest PR of 94.10±2.45 % was observed at a flow rate of 100 mL/min. Beyond the flow rate of 100 mL/min, the PR steeply decreased. This is majorly attributed to the intense turbulence

caused by high air flow rate. Previous studies conducted on LBS observed a similar trend [44,146,264]. However, the SE plunged after 100 mL/min. The highest SE of 98.82±2.18 % was observed at 125 mL/min. For lipids, the highest extraction yield of 65.99±0.75 % was observed at an air flow rate of 100 mL/min. A similar trend to PR was obtained with lipid yield. After 100 mL/min, the lipid yield reduced abruptly. The statistical analysis by One-Way ANOVA revealed that the data obtained from this study differed significantly ($p<0.05$). Therefore, for further experiment, air flow rate of 100 mL/min was selected.

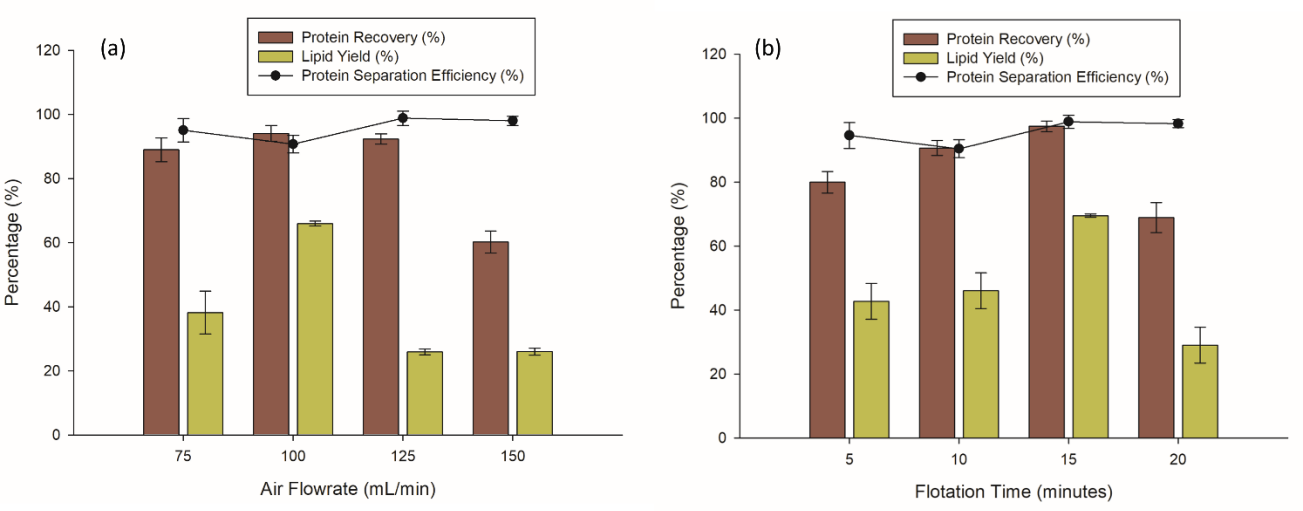


Figure 4.4 PR, PSE and LR with respect to a) flotation air flow rate and b) flotation time of salt solution on U-LTF system.

The second crucial SS parameter of flotation time was manipulated in this study. The range of flotation time selected was from 5 min to 20 min. The results of this study are depicted in Figure 4.4b. It is evident that at 15 min of flotation, the highest PR and LR of 97.43±1.67 % and 69.50±0.54 %, respectively were observed. The highest protein SE of 98.88±2.07 % was observed at 15 min of flotation. At a flotation time of 5 min, the PR and LR were very low due to insufficient mass transfer of proteins and lipids to the respective phases. Although at 20 min of flotation, there was a sharp decrease in PR

and LR. This is contributed by the oxidation and accumulation of various biomolecules in the LTF system [260]. Additionally, prolonged flotation duration results in the dissolution of lipids and proteins in other phases thus resulting in poor biomolecule recovery [311]. The statistical data analysis revealed that results obtained in this study differed significantly ($p<0.05$). Thus, a flotation time of 15 min was selected as the optimum settings for biorefinery of microalgae *via* LTF system.

4.3.4. Comparison between U-LTF, TPP and LTF

The optimum setting of U-LTF system was compared with the control LTF without the cell-disruption technique and conventional TPP system. The results obtained from this study are tabulated in Table 4.2. U-LTF system provides higher PR as well as LR compared to LTF and TPP. The absence of cell-disruption method in both these systems jeopardizes the release of biomolecules to the surrounding solution. Additionally, TPP requires the highest amount of energy due to intense stirring and centrifugation as well as consume more time for the entire process. Whereas LTF system equipped with ultrasonication ensures effective release of cell contents while flotation of these biomolecules to respective phases. The data obtained from this study were subjected to One-Way ANOVA analysis and differed significantly ($p<0.05$).

Table 4.2 Results of comparison and recycling study of TLF system.

Comparison Study			
	Protein recovery	**Protein separation efficiency**	**Lipid recovery**
TPP	70.93±2.98 %	89.56±1.87 %	56.02±3.76 %
LTF	82.10±2.14 %	87.11±3.79 %	63.88±1.89 %
U-LTF	97.43±1.67 %	98.88±2.07 %	69.50±0.54 %
Recycling Study			

	Protein recovery	Protein separation efficiency	Lipid recovery	Alcohol purity
1st run	97.43±1.67 %	98.88±2.07 %	69.50±0.54 %	99.9 %
1st recycling	92.02±0.79 %	91.73±1.71 %	62.42±1.84 %	92.5 %
2nd recycling	90.67±2.36 %	88.17±3.51 %	59.39±0.54 %	88.2 %

4.3.5. Effect of recycling on LTF system

For this study, the effect of recycled top phase (t-butanol) was investigated. This study is important as a high performance of LTF system with recovered t-butanol will ensure lower operating cost and low waste generation by this technology. This study analysed the recycling of t-butanol for 3 cycles and its respective PR and LR. The results from this study are tabulated in Table 4.2. From the results, with further recycling cycles, the purity of t-butanol decreases and thus affecting protein and lipid recovery. However, additional top-up of alcohol with each cycle can be implemented to obtain superior results [263,312]. Thus, proteins extracted from U-LTF can be further processed as feed for fish, animals and poultry. Whereas lipids extracted in the top phase can be potentially utilised for biodiesel production.

4.4. Conclusions

Extraction of lipid and protein from *Chlorella sorokiniana* by utilising U-LTF system was investigated in this study. The optimum parameters recorded were microalgal biomass loading of 0.5 w/v%, $(NH_4)_2SO_4$ concentration of 40 w/v%, a volume ratio of 1:1.5 (salt:alcohol), ultrasonication pulse mode of 20s ON/20s OFF at 20 % amplitude for 5 min, flotation air flow rate of 100 mL/min and flotation time of 15 min. The corresponding protein and lipid recovery of 97.43±1.67 % and 69.50±0.54 % were observed. A comparison study with TPP and control LTF system revealed that protein and lipid recovery of ultrasonication-assisted LTF system were higher by around 26 % & 12 %

and 15 % & 7 % respectively. This provides a positive insight into the potential utilisation of U-LTF system for biorefinery of *Chlorella sorokiniana* to promote a circular economy.

CHAPTER 5

5. Liquid triphasic systems as a sustainable downstream processing of *Chlorella* biorefinery for potential biofuels and feed production

5.1. Introduction

Climate change and global warming have driven the conventional fossil fuel-based energy sector towards more renewable and sustainable sources of energy. In the past century, a lot of research has been focused on harnessing conventional naturally occurring sources of energy such as solar, wind, hydrothermal, geothermal, and hydropower. However, these renewable energy plants require huge capital as well as higher expenditure on equipment maintenance [313]. Moreover, the lifecycle analysis of these plants doesn't result in a high net positive value due to the high resources required for their fabrication/construction [314]. These sources of energy are also not available throughout the year and are seasonal depending on environmental conditions. Geographic limitations exist in almost every location when a continuous supply of energy is required and storage costs of this form of energy are also very high. For example, the storage of solar energy harvested needs to be stored in high-capacity batteries which require very high capital. Thus, a cheaper, eco-friendly, sustainable, and perennial alternative is necessary to combat these difficulties [315].

Apart from the sources of clean energy, biomass has also been a potential candidate for obtaining fuel. Biomass-derived from oil crops has been mainly focused on the first and second generation of biofuels. Although, there were several drawbacks such as high natural source (water, fertile land) demand combined with low yield. But this paved the way for better and high-yielding third-generation biofuels mainly derived from algae. Compared to oil crops such as soybeans, oil palm utilised in first-generation, algae do not compete with arable land and freshwater necessary for food crops. Additionally, algae are excellent at up taking atmospheric or even

anthropogenic industrial flue gas CO_2 as a source of carbon. Algae thrive on wastewater, such as sewage, paper mill, or other industrial wastewater, as it provides the essential nutrients fundamentally necessary for multiplication. Some species such as *Sargassum* and *Phaeophyceae* can tolerate seawater salinity and grow in extreme conditions. This wide spectrum of environmental conditions provides external stress on algae and results in the higher secretion of specific metabolites as a response. Singh *et al.* concluded that a temperature of 35ºC and illumination of 420 µmol m^{-2} s^{-1} resulted in an increased biomass growth rate of 1.73 d^{-1} of *Selenastrum minutum* [316]. Algae such as cyanobacteria, red, brown, blue-green, microalgae also secrete secondary metabolites and other bioactive components as part of their cell body. These metabolites such as proteins, lipids, and carbohydrates can be further extracted to obtain feed for poultry, fishes, animals, and humans. Microalgae are reported to produce clean and safe polyunsaturated fatty acids compared to fishes who are more prone to toxicity due to polluted oceans [317]. Different species of microalgae secrete different spectra of pigments according to the illuminance stress and type of species [318]. *Haematococcus pluvialis* secretes red pigment astaxanthin, *Spirulina* spp. and *Chlorella* spp. secrete green pigment chlorophyll. These pigments are widely utilised in numerous applications such as cosmetics, consumables, and medicines. Microalgae such as *Spirulina platensis* and *Chlorella vulgaris* have been circulated in the industry as a potential source of protein. The biomass from these microalgae is utilised as feed for humans, animals, fishes and birds [40].

Microalgae essentially have superior properties and numerous advantages such as rapid growth and utilisation of natural resources such as sunlight, atmospheric CO_2, but they are also composed of low concentration in culture broth and repelling charges on their cell wall. This limits their agglomeration for water separation [33]. A lot of energy is required for dehydration, which is usually carried out by physical methods such as

centrifugation or chemical methods such as flocculation [319]. Microalgae have a rigid cell wall that needs to be broken down to release all the metabolites in the surrounding environment, thus a cell-disruption pre-treatment method is crucial for higher extraction [320]. These two processes are major drivers of high costs related to industrial-scale utilisation of microalgae as a source of biofuel. There are two pathways to tackle these issues: either reducing the energy consumption related to cell-wall disruption and water dehydration or extracting multiple products with a biorefinery concept [321]. As the current commercial processes mainly focus on the extraction of single components, while the resultant biomass is either recycled as a source of energy or discarded, most of the microalgal biomass is under-utilised.

This study focuses on incorporating the second option of biorefinery to tackle the major bottleneck of large-scale microalgae-based biofuel generation. Phase separation processes, such as ATPS and TPP system have been widely used in the extraction of numerous compounds from biomaterials such as lipase, betacyanin, proteins, enzymes [275,312]. These processes utilise the mixing of two immiscible solvents/polymers or aqueous solutions to form two or more layers in the system with a stable interface separating them [322]. This interface acts as a gateway for the exchange of biomaterials between phases. Addition of flotation or SS to ATPS to form a LBF system has been examined to extract high yields of bacteriocin (70 %), lipase (99.2 %), betacyanin (95 %) and proteins (88.6 %) from *Pediococcus acidilactici Kp10*, *Burkholderia cenocepacia* ST8, *Hylocereus polyrhizus* and *Chlorella vulgaris*, respectively [260,263]. The flotation bubbles provide a platform for the bio-components to transport from one layer to another which is usually achieved by centrifugation. The LBF system is beneficial as it offers a high extraction yield and concentration coefficient. Although being easier to handle, consuming less space and time, and being able to operate continuously, the LBF system can only extract a single

component in the top phase. TPP is a three-phase forming system utilised to extract and purify the compounds. There are numerous studies for the extraction of proteins and lipids from algae with TPP [323–326]. Thus, the TPP system is incorporated with SS to obtain the LTF system. The three phases consist of the bottom salt phase, middle interface protein precipitate and top organic phase. In the TPP system, t-butanol is generally utilised as organic phase in TPP system as it exhibits low flammability and high boiling point for phase recycling [327].

The current study focuses on integrating ultrasonication with TPP and SS to form a U-LTF system. This U-LTF system was utilised for biorefinery of microalgae *Chlorella sorokiniana* CY-1 to extract lipids, proteins, and carbohydrates in the respective phases. The ultrasonication assists in efficient cell-wall disruption for higher extraction. Numerous parameters were manipulated to optimise the system and control studies were performed to compare this system. The phases were recycled, and the sustainable cycling nature of the system was tested. The GC-FID profile of FAME in the upper phase and the Fourier transform infrared spectroscopy (FTIR) profile of carbohydrates in the bottom phase were analysed. This study can potentially unlock a successful biorefinery approach of microalgae for the feed and fuel industry.

5.2. Experimental

5.2.1. Materials

$(NH_4)_2SO_4$, t-butanol, NaOH, Bradford's reagent, methanol, tris-HCl buffer, sulfuric acid, phenol, and chloroform were procured from R&M Chemicals. All these chemicals and solvents were of analytical grade.

5.2.2. Microalgae cultivation for bioenergy and biofuel production

This study utilised *Chlorella sorokiniana* CY-1 which was sourced from National Cheng Kung University, Taiwan. The microalgae strain cell composition was 40 % of protein, 30–38 % of carbohydrates and 18–22 % of lipids [300]. For the cultivation of microalgal

culture broth, a pre-culture (200 mL) was prepared with BG-11 medium as described in the Chapter 4. Once it reached maturation, the pre-culture was transferred to batch culture (1 L) with the same medium composition in an enclosed laboratory-scale PBR. In the PBR, the culture was equipped with aeration of a mixture of air and CO_2 (5 v/v%) at 400 mL/min, irradiation of 200 μmol/m^2/s, and agitation of 450 rpm. The biomass growth was monitored, and harvest of culture broth was conducted by centrifugation at 4960 g (5 min) once it reached maturation. The wet cell pellets were freeze-dried for 36 h and resultant biomass powder was stored at −20 °C. The experiments were carried out in 2 weeks.

5.2.3. Determination of microalgae cell concentration

The culture broth was observed daily for its cell concentration through optical density. A 2 mL sample was collected from the culture and diluted 10 x followed by analysis with UV–Vis spectrophotometer (UV-1800, Shimadzu) at 680.8 nm of wavelength.

5.2.4. Equipment set-up of LTF system

The equipment utilised in this experiment was fabricated and sourced from Donewell Resources (Malaysia). The equipment set-up was similar as described in Chapter 4.

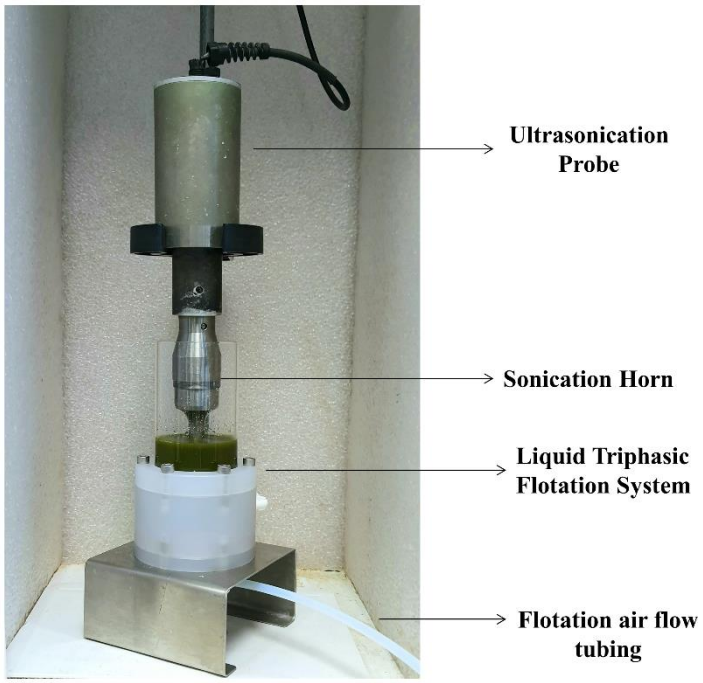


Figure 5.1 Set-up of U-LTF system.

5.2.5. Operation and optimisation of U-LTF system

At the beginning of each run, enough air flow was supplied to the equipment to ensure no backflow of solvents. For the preparation of the bottom phase, microalgal biomass was mixed with 50 mL of selected concentration of $(NH_4)_2SO_4$ solution. For the top phase, 50 mL of 100 % t-butanol was added. After top-up with alcohol, flotation was introduced at an airflow rate of 20-25 mL/min for at least 5 min. As the system stabilized, the flowmeter was adjusted to the selected airflow rate for the desired duration. Simultaneously, cell-wall disruption was conducted by an ultrasound probe (Bandelin Sonopuls UW 2200, Germany) was immersed in the system. The sonicator probe was fitted out with titanium horn sonotrode (TT 13/FZ) and the amplitude was set at 20 % with a pulse mode of 10 s ON/ 20 s OFF for 5 min at room temperature. The maximum temperature was 35 ºC and no external bath was necessary. The flotation was run for selected time duration and all three phases were collected at the end of each run. Their volume was noted, and analysis was performed. OVAT approach was taken for parameter

optimisation in this study. Table 5.1 lists down all the parameters employed in this system. The parameters were selected based on the recovery of proteins, carbohydrates, and lipids and protein SE. The middle protein precipitate was collected and 0.1 M Tris-HCl buffer was added prior to analysis. The bottom, middle and top phases were utilised for analysis of proteins. For lipids analysis, the top phase was utilised, and the bottom phase was analysed for carbohydrates. The analysis of all the samples was conducted in triplicates.

Table 5.1 Initial settings of U-LTF system.

Parameters	Initial settings	Manipulated settings	Units
$(NH_4)_2SO_4$ concentration	40	30, 35, 40, 45, 50	w/v%
Volume ratio	1:1	1:0.5, 1:1, 1:1.5, 1:2	salt:t-butanol
Air flowrate	100	50, 100, 150, 200	mL/min
Flotation duration	10	5, 10, 15, 20	min
Ultrasound duty	10/20	5/10, 10/20, 20/30, continuous	s ON/ s OFF
pH	5.25	4.75, 5.25, 6.5, 7.8	NA

5.2.6. Analysis of algal components

5.2.6.1. Proteins

The analysis of proteins extracted by LTF was conducted by Bradford's method. In this assay, 0.25 mL of sample was added to 2.5 mL of Bradford reagent and the absorbance was read at 595 nm with UV-Vis spectrophotometer after 10 min. The concentration of proteins was calculated by comparing the absorbance of the sample with a BSA standard calibration curve. The BSA concentration for calibration curve was between 0-1.2 g/L.
The analysis of proteins was performed by assessing the SE and recovery for subsequent parameter optimisation. PSE was defined as yield of protein in terms of concentration in the top phase. It was determined by equation 4.1 as described in the previous section.

The PR was computed by equation 4.2. The calculation for P_i was conducted in the previous section.

5.2.6.2. Lipids

For the analysis of lipids, the top phase was vaporized with a rotary evaporator with water bath of 40 ºC and vacuum pressure of 96 mbar (EYELA) and the remaining contents were obtained. The identification of lipids in this sample was done by the modified Folch method [301,322]. The residual lipids were saponified in 4 mL of 25 % methanolic NaOH mixture at 100ºC for 30 min. The sample was left to cool down and was added to 6 mL of chloroform:methanol solution in the ratio 2:1. This mixture was vortexed for 2 min and subsequently spun at 12,000 g for 2 min. The supernatant lipid mixture was pipetted out and dried under N_2. The total weight of the dried lipids after extraction was noted. The yield of lipids was calculated by comparing the dried weight of the lipids sample with the dried weight of initial lipids in microalgae. The lipids recovery (LR) was calculated by following equation (5.1).

$$LR\% = \frac{weight\ of\ lipids\ in\ organic\ phase}{total\ weight\ of\ lipids\ in\ microalgae\ biomass} \times 100\ \% \qquad \text{(Eq 5.1)}$$

The GC-FID assay on lipids was conducted according to the protocols stated by Patel *et al.* [329]. To the extracted lipids, hexane and water were added to obtain fatty acids methyl esters. 10 µl of 1 mg/mL of Nonadecanoic acid (C19:0) was added as an internal standard. The upper phase was transferred to a clear GC *via*l for further analysis. A Gas Chromatograph (Perkin Elmer Clarus 690 GC, USA) equipped with a flame ionization detector was used to test the samples. The column was obtained from Agilent Technologies (30 m ×0.25 mm × id 0.25 µm) and the samples were run with helium as a carrier gas at 1.0 mL/min. The column oven temperature was set at 35 ºC for the first 3 min and eventually rose to 220 ºC with an increment of 4 ºC/min. The temperature was maintained at 220 ºC for 35 min. The temperatures of detector and injector were

set at 260 ºC and 250 ºC, respectively. The split ratio was 15:1, carrier gas (helium) was controlled at 103.4 kPa.

5.2.6.3. Carbohydrates

The total carbohydrates analysis for the bottom phase was conducted by the phenol-sulfuric method. The salt solution in the bottom phase was precipitated out with twice the amount of methanol. This mixture was further filtered to remove the crystalline salt from the samples [330]. For 2 mL of the filtered sample, 1 mL of 5 v/v% phenol was added and vortexed. Then 5 mL of 96 v/v% sulfuric acid was added and vortexed. The sample was kept aside for 10 min followed by 20 min of water bath at 20-30ºC. Then absorbance of the sample with UV-Vis Spectrophotometer at 490 nm was noted [331]. A standard calibration curve with glucose was prepared for comparison with the sample. Carbohydrates recovery (CR) was calculated by the following equation (5.2)

$$CR\% = \frac{carbohydrates\ content\ in\ the\ bottom\ phase}{total\ carbohydrates\ content\ in\ microalgae\ biomass} \times 100\ \% \qquad \text{(Eq 5.2)}$$

The carbohydrates obtained at the bottom phase were characterized by FTIR. The transmittance mode was selected to characterize the presence of carbohydrates specific chemical groups in the obtained sample. The sample obtained post-salt precipitation was used and IR spectra were obtained *via* FTIR spectrophotometer (Frontier, Perkin Elmer) with a wavelength range of 4000–400 cm^{-1} at an average of 32 scans.

5.2.7. Recycling, large-scale and control experiments

The recycling ability of the proposed system was performed by reusing both the top and bottom phases after extraction. The top phase was evaporated in a rotary evaporator and recycled t-butanol was obtained. The purity of the recycled t-butanol was analysed by a digital refractometer (ATAGO). The resulting Brix No. was computed and compared with the calibration curve for t-butanol. This recycled alcohol was further utilised for the second extraction cycle. This process was repeated at least three times to observe the recycling nature of the system. For recovery of the salt, the bottom phase was mixed

with twice the amount of methanol to precipitate $(NH_4)_2SO_4$. Further filtration was utilised to obtain crystallized $(NH_4)_2SO_4$. This recovered salt was further utilised for the next cycle of extraction. The recovery of both the alcohol and salt was noted.

The large-scale study of this system was carried out by scaling up U-LTF 15 times to the current volume. The working volume was fixed at 1.5 L. All the optimised parameters were used and only the volume of the phases was scaled up. The corresponding PR, PSE, LR, and CR were calculated and compared with the small-scale study.

The TPP comparison study was conducted by utilising U-LTF parameters. In this experiment, flotation was not carried out. Two sets of TPP studies (U-TPP and TPP) were carried out with U-TPP utilising ultrasonication. For TPP experiments, the system was stirred at 200 rpm for 1 h and was settled for another hour. A third control set without ultrasonication, *viz*. LTF, was carried out. The results of these control studies are listed in the next section. The experiments were conducted in triplicates.

5.2.8. Statistical analysis

All the data collected in this study were analysed for One-Way ANOVA with a two-tailed *t*-test. The data mentioned in this paper was written as mean ± standard de*via*tion. The statistical analysis of the data obtained from this study was conducted by Microsoft Excel to evaluate the sum of differences with significance $p \leqslant 0.05$.

5.3. Results and Discussion

5.3.1. Effect of salt concentration and alcohol volume ratio

The alcohol and salt required for this experiment were t-butanol and $(NH_4)_2SO_4$, respectively. The concentration of the salt solution in this system was manipulated to observe the change in the proteins, lipids, and CR. The salt concentration was changed from 30 w/v% to 50 w/v% and the corresponding analytical results were calculated [305,326]. According to Figure 5.2a, the recovery of all the three components peaked at a salt concentration of 40 w/v%. The PR and PSE increased between salt concentration

of 45-50 w/v% but it was still lower than 74.93±5.47 % observed at 40 w/v%. Overall, PR and PSE are highest at an $(NH_4)_2SO_4$ concentration of 40 w/v%, which is in accordance with the previous studies conducted [326,328]. The LR exhibited a similar trend as PR, it peaked at a salt concentration of 40 w/v% with the corresponding value of 40.69±4.03 %. Gaur *et al.* conducted a TPP study for oil extraction from rice bran, mango kernel, and soybean. The study concluded that the highest LR is observed at a salt concentration of around 30-40 w/v% [332]. A similar trend was observed by Shah *et al.* with the extraction of oil from Jatropha seeds *via* enzyme-assisted TPP [333] and observed a comparable outcome of CR to that of LR except for a low recovery at 35 w/v%. The highest CR was observed at 40 w/v% with a recovery of 40.08±2.37 %. Varakumar *et al.* studied the extraction of gingerols and shogaol from ginger rhizome powder and concluded that the optimum recovery of these carbohydrate phenolic components occurred between 40-45 w/v% [324]. Mondal *et al.* also confirmed high recovery of starch *via* TPP with an $(NH_4)_2SO_4$ concentration of 40 w/v% c Therefore, $(NH_4)_2SO_4$ concentration of 40 w/v% was selected for further optimisation studies.

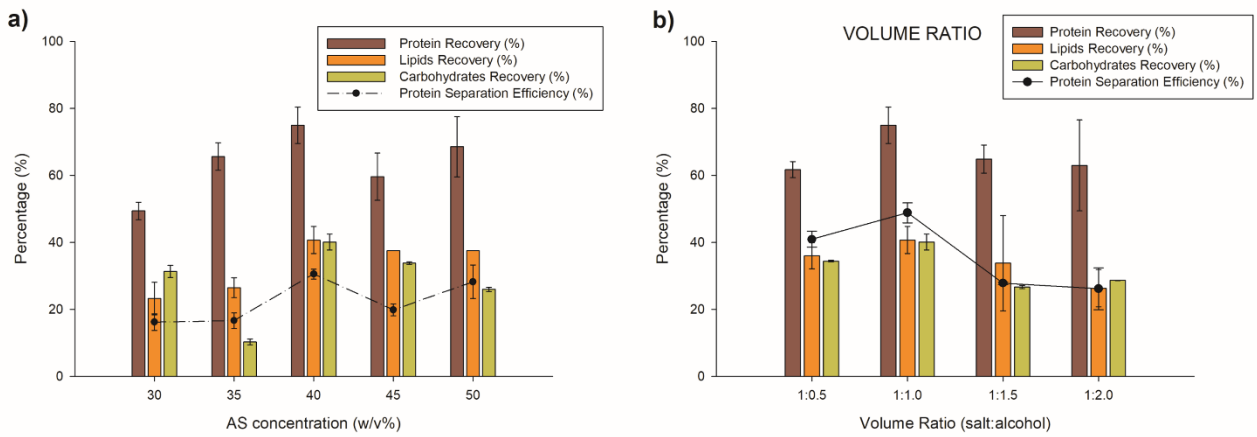


Figure 5.2 PR, LR, CR, and PSE of a) $(NH_4)_2SO_4$ concentration and b) volume ratio on U-LTF system.

In this part of the section, the volume ratio of alcohol:salt solution was manipulated. The salt solution volume was kept constant at 50 mL, while the alcohol volume was altered with a ratio from 1:0.5 to 1:2. Figure 5.2b depicts the effect of the salt to alcohol volume ratio on the performance of component recovery. A volume ratio of 1:1 gives the highest PSE and PR of 48.82±3.03 % and 74.93±5.47 % respectively. With an increase in the volume of t-butanol, protein denatures and hinders precipitation of these proteins; thus a decreasing trend was observed [304]. LR peaks at a volume ratio of 1:1 with a corresponding value of 40.69±4.03 % as well with 1:0.5 an immediate second at 36.01±3.87 %. Mulchandani *et al.* reported a similar observation while extracting lipids from *Chlorella saccharophilia* and concluded that an increase or decrease in the volume ratio of t-butanol does not result in higher lipid recovery as the amount of $(NH_4)_2SO_4$ salt might not be sufficient for stronger phase separation [335]. For CR, a volume ratio of 1:1 observed the highest CR of 40.08± 2.37 % followed by 34.40±0.24 % at a volume ratio of 1:0.5. Sharma *et al.* observed a similar pattern for extraction of carbohydrate polymers by TPP with the highest CR occurring at a volume ratio of 1:1 and concluded that change in the volume ratio does not affect the precipitation of polymers [336]. The statistical analysis conducted reveals that the differences in the data was significant as denoted by p-value <0.05. Thus, a volume ratio of 1:1 was selected for further optimisation of other parameters.

5.3.2. Effect of flotation air flowrate and time

The U-LTF system provides an advantage over the conventional U-TPP system with respect to the capability of flotation for faster and quicker mass transfer [311,328]. This section focused on manipulating the two crucial parameters of flotation air flow rate and time with previously selected salt concentration and volume ratio. Previously reports showed that high flotation air flow rate resulted in turbulent flow of the air bubbles thus resulting in low targeted mass transfer [337]. An air flow rate of 50 mL/min resulted in

high PSE and PR as well, although a slight increase in air flow rate resulted in a higher recovery. In this study, an air flow rate of 100 mL/min resulted in the highest PSE and PR of 30.56±1.47 % and 74.93±5.47 %, respectively. A similar trend was reported in previous studies [263,264]. For lipids and CR, a much simpler pattern was observed with the highest peak at 100 mL/min (Figure 5.3a). The highest LR was observed as 40.69±4.03 % and corresponding CR of 40.08±2.37 %. Both lipids and carbohydrates graph observed second highest recovery at 150 mL/min with corresponding values of 37.36±1.97 % and 24.31±0.09 % respectively. As the higher flow rate of air results in increased energy demand, this setting was not considered. The statistical analysis conducted disclosed that the differences in the data was significant as denoted by p-value <0.05. Therefore, a flotation flow rate of 100 mL/min was selected for further optimisation of flotation time.

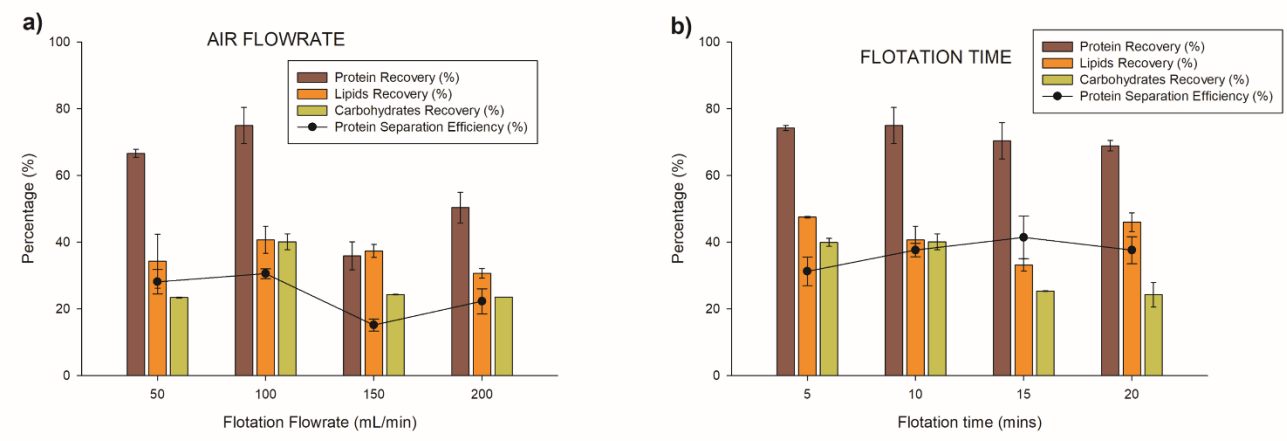


Figure 5.3 PR, LR, CR and PSE of a) flotation air flowrate and b) flotation time on U-LTF system.

Flotation time was altered from 5 to 20 min to observe the highest PSE, PR, LR and CR. According to Figure 5.3b, PR was comparable at flotation time of 5 and 10 min with corresponding values of 74.18±0.78 % and 74.93±5.47 %, respectively. The values for PSE, on the other hand, peaked at a flotation time of 15 min but the recovery of total

proteins was low. It can be concluded that a prolonged flotation time did not result in higher PR, which is mainly attributed to the fact that mass transfer of proteins reached equilibrium in the U-LTF system and additional flotation will lead to oxidation of the metabolite [260]. The extended flotation duration also resulted in the dissolution of proteins in other phases [311]. As for lipids, a flotation time of 5 min resulted in the highest recovery of 47.47±0.26 % followed by 20 min at 45.96±2.84 %. The LR profile indicated in Figure 5.3b clearly states that a flotation time of 5 min was enough for the mass transfer of lipids and further flotation was not necessary. However, carbohydrates followed a similar trend with PR. The CR at 5 min flotation time was 39.92±1.19 % compared to 40.08±2.37 % at 10 min flotation. There was a slight increase in CR when the flotation was prolonged; however, doubling the time from 5 min to 10 min means additional expenditure. Therefore, a shorter duration of 5 min was preferred and selected for further optimisation studies. The statistical analysis conducted disclosed that the differences in the data was significant as denoted by p-value <0.05.

5.3.3. Effect of ultrasound duty cycle and pH

The optimised flotation parameters were utilised for the further parameter of ultrasound duty. Previous studies indicated that an ultrasonication duration of 5 min is sufficient for efficient cell disruption [338]. Extended exposure of ultrasound waves results in the disintegration of cells and an increase in overall system temperature, which denatures the extracted components. This increase in temperature was mainly attributed to the transformation of sound energy to heat energy [308]. Figure 5.4a depicts the results for continuous ultrasonication and pulse modes. An increase in the ratio of the ultrasound cycle resulted in higher recoveries of PR, LR and CR. The PR at pulse mode of 20s/30s was higher to continuous cycle with corresponding values of 77.75±4.25 % and 74.07±7.69 % respectively. The PSE peaked at pulse mode of 20s/30s. Khoo *et al.* conducted an ultrasound-assisted LBS for extraction of astaxanthin from *Haematococcus*

pluvialis and observed a similar trend with a comparative yield at continuous cycle and pulse cycle of 30s/5s [339]. Compared to PR, LR and CR followed an obvious pattern with the highest LR and CR of 47.69±4.84 % and 42.99±0.97 %, respectively, at pulse mode of 20s ON/30s OFF. Panadare *et al.* observed a similar trend with most of the lipids recovered by 5 min [305]. The statistical analysis conducted disclosed that the differences in the data was significant as denoted by p-value <0.05. Therefore, ultrasound setting of pulse mode with 20s ON/ 30s OFF was selected for further analysis of pH.

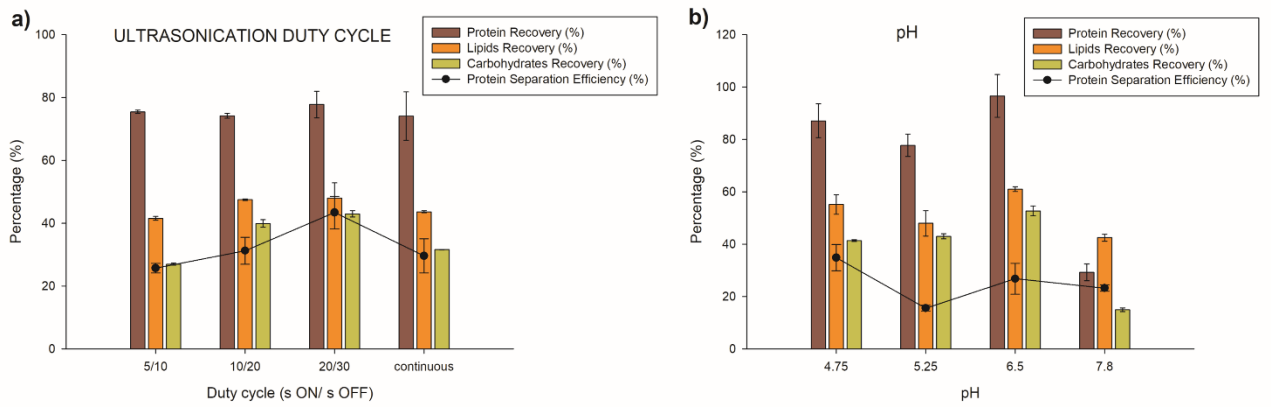


Figure 5.4 PR, LR, CR and PSE of a) ultrasonication duty cycle and b) pH on U-LTF system.

The adjustment of pH is a crucial factor in optimising the U-LTF system. Change in pH affects the ionization of the basic or acidic amino acids of protein and changes their net surface charge thus altering the partition behaviour. In this study, pH was manipulated from 4.75 to 7.8 by adding either NaOH or HCl. The results are depicted in Figure 5.4b, showing that the isometric point of protein was achieved in a slightly higher pH value of 6.5. A strong acidic pH of 4.75 results in high PSE and PR as well, while it is lower than the corresponding values at pH 6.5. With alkaline pH of 7.8, low PSE and PR were observed probably due to the higher solubility of proteins in the aqueous bottom phase. With pH higher than the isometric point, the net charge on proteins shifts to a negative

value thus resulting in protein accumulation at the bottom phase [325]. The highest PR of 96.59±8.15 % was thus obtained at pH of 6.5. For the case of LR, a similar principle was applied. The highest recovery of lipids was observed at the isoelectric point (pI) corresponding to a pH of 6.5. Previous reports also suggested that the average pI of oils from different sources is observed in the range of 5.7-6.6 [305]. In this study, the highest LR of 61.02±0.91 % was observed at a pH of 6.5, which was in the acceptable range of the previous studies. Qiu *et al.* also reported that the highest lipids in the form of total fatty acids were observed at pH 6-7 [306]. CR was comparable at pH values of 4.75 and 5.25 with a corresponding value of 41.39±0.37 % and 42.99±0.97 %, respectively. Although the highest recovery was observed at pH 6.5 with a corresponding value of 52.69±1.90 %. Sharma *et al.* reported a similar trend for extraction of chitosan with the highest recovery obtained at pH 7 [340]. Pakhale *et al.* concluded that this phenomenon might be related to better structural confrontation stability of carbohydrates towards the alcohol [341]. The statistical analysis conducted disclosed that the differences in the data was significant as denoted by p-value <0.05. Therefore, a pH value of 6.5 was selected as the final optimised parameter, and different control studies were conducted.

5.3.4. Control studies with LTF, U-TPP, and TPP

The above-optimised parameters were incorporated to compare the control studies where ultrasound and flotation were absent. The control studies are the most widely practiced techniques for the extraction of biomolecules *via* phase separation. Three different sets of control studies were conducted, and the results are depicted in Figure 5.5. As expected, there is a decreasing trend with each PR, LR and CR. However, the LR does not markedly decrease across the control studies. The statistical analysis conducted disclosed that the differences in the data was significant as denoted by p-value <0.05.

The results also prove that the addition of flotation and ultrasound steps benefit the existing TPP system.

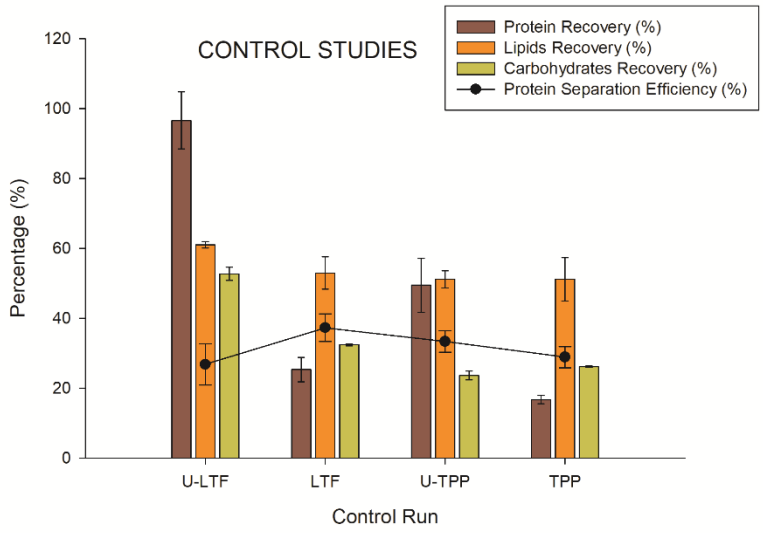


Figure 5.5 PR, LR, CR, and PSE of control studies on U-LTF system.

5.3.5. Large-scale operation and the recovery of alcohol and salt

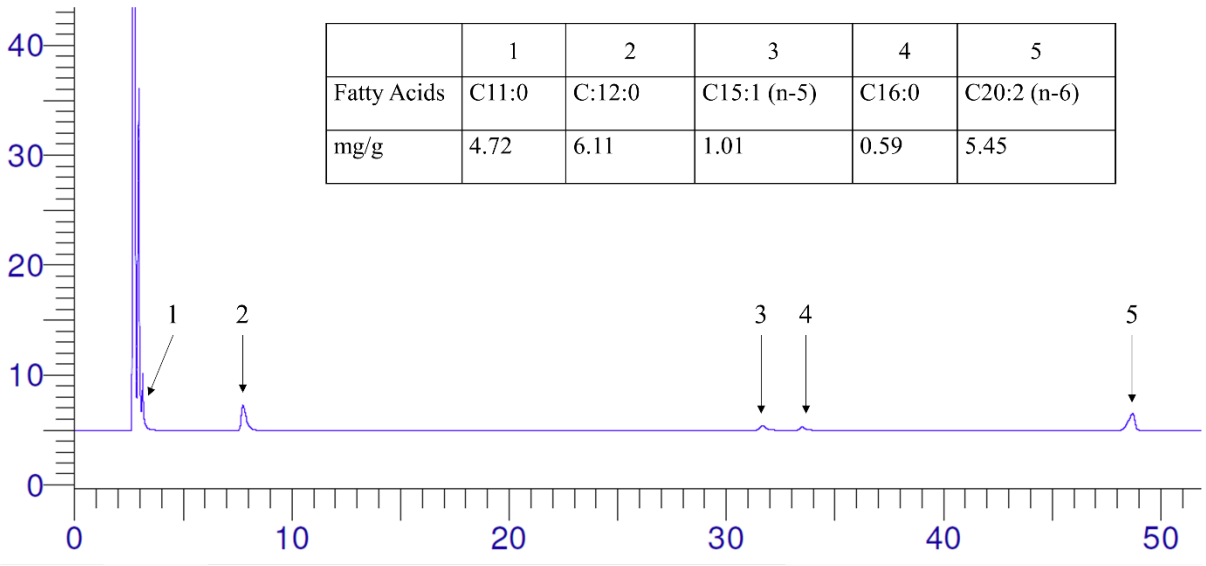
Apart from control studies, the laboratory-scale U-LTF system of 100 mL was scaled-up to a working volume of 1.5 L (15-fold increase). The resulting values of PSE, PR, LR and CR were 33.27±2.00 %, 92.81±4.20 %, 58.97±0.76 % and 48.89±0.87 % respectively. It is worth noting that an increase in the scale of the experiment did not affect the recovery performance and consistent analytical results were obtained. The phases involved in this experiment were also recovered by evaporation and precipitation followed by crystallization. As shown in Table 5.2, the recovery of both alcohol and salt decreases after each cycle, and the purity of both phases also reduces. This can be corrected by topping-up fresh stock of both components to maintain the purity and quantity [261,263].

Table 5.2 Recycling studies of U-LTF system.

Recycling	Protein separation efficiency (%)	Protein recovery (%)	Lipids recovery (%)	Carbohydrates recovery (%)	Alcohol recovery (%)	Alcohol purity (%)	Salt recovery (%)
1st Run	32.97±8.49	96.59±8.15	61.02±0.91	52.69±1.90	NA	99.9	NA
1st Recycling	31.06±1.89	91.87±3.15	58.97±0.76	48.89±0.87	96	96.4	94
2nd Recycling	32.81±0.97	89.79±5.78	55.35±1.82	45.52±1.67	92	93.1	91
3rd Recycling	30.52±1.83	85.48±2.21	51.42±1.36	42.48±1.94	86	86.8	85

5.3.6. Application of microalgae in bioenergy and biofuel

The extracted top and bottom phases were analysed *via* GC-FID and FTIR to confirm the presence of lipids and carbohydrates. Figure 5.6 summarizes the results from the GC-FID analysis. Five peaks of methyl esters were reported mainly from middle chains (C11:0, C:12:0, C15:1 n-5, C16:0) and long-chain (C20:2 n-6). As shown in Figure 5.6, the middle chains of undecanoic acid and decanoic acid (lauric acid) comprise the highest proportion with a weight content of 4.72 mg/g and 6.11 mg/g. The long chain of Eicosadienoic acid (C20:2 n-6) also showed a high weight fraction of 5.45 mg/g. Hexadecanoic acid or Palmitic acid (C16:0) had a value of 0.59 mg/g. Yew *et al.* studied a hybrid LBS for extraction of lipids from *Chlorella* sp. and obtained a similar yield of palmitic acid [342]. Gharibeh *et al.* investigated the ignition and combustion characteristics of FAME middle chains and concluded that an increase in the carbon atoms in these chain lengths corresponds to higher fuel reactivity but is limited to a certain threshold value [343].



	1	2	3	4	5
Fatty Acids	C11:0	C:12:0	C15:1 (n-5)	C16:0	C20:2 (n-6)
mg/g	4.72	6.11	1.01	0.59	5.45

Figure 5.6 GC-FID spectrum of lipids.

FTIR spectroscopy was conducted on the extracted carbohydrates in the bottom phase. Figure 5.7 depicts the FTIR spectrum for the bottom phase of the U-LTF system. The stretching observed near 3350 cm^{-1} was due to the stretching of OH bonds in the primary carbohydrates such as glucose. The asymmetric vibration at 2945 cm^{-1} and symmetric vibration at 2833 cm^{-1} are attributed to CH bonds in the carbohydrates chain. The strong transmittance at 1018 cm^{-1} was due to the vibration of CO and CC bonds. Therefore, the FTIR spectrum suggests that the bottom phase has suitable carbohydrates, which can be utilised for producing bioethanol *via* fermentation.

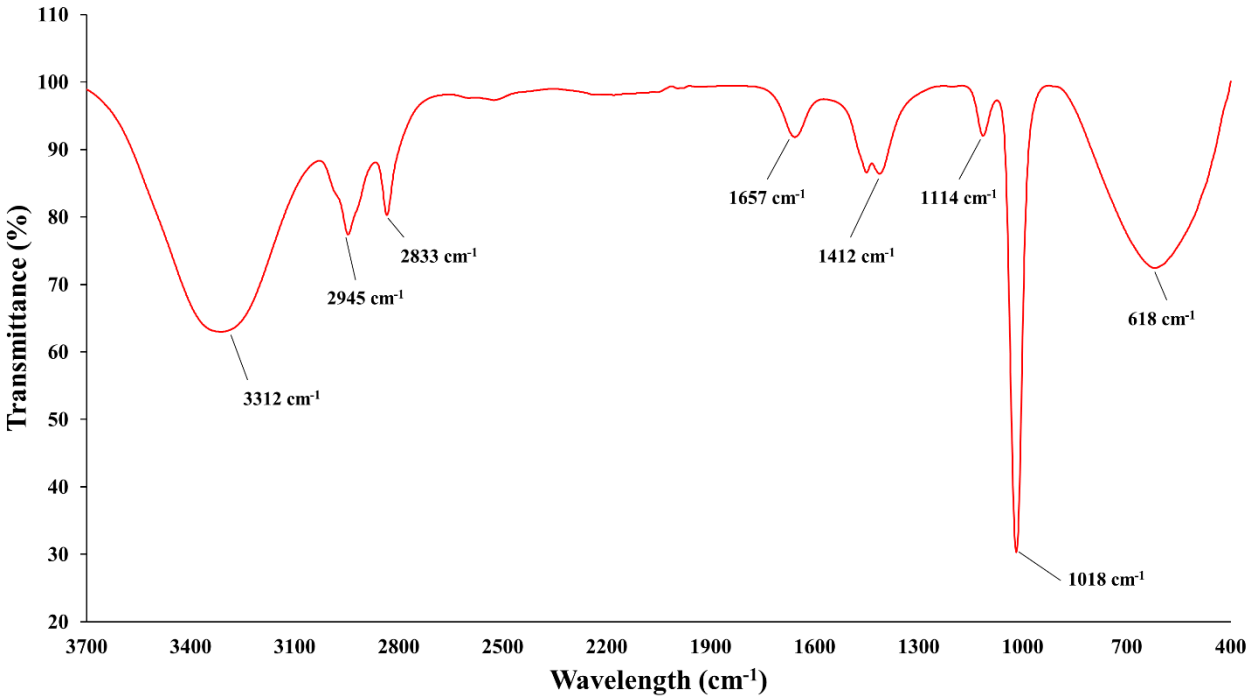


Figure 5.7 FTIR spectrum of carbohydrates.

The analytical methods described in this section, confirm the presence of carbohydrates and lipids in the extracted phases. This proves that, upon further purification, these components can be utilised for efficient and low-cost bioenergy production due to the biorefinery nature of the U-LTF system. Future research should focus on purifying these components and optimising the life cycle cost.

5.4. Conclusions

This study focused on optimisation of different parameters of U-LTF system to extract proteins, lipids and carbohydrates from *Chlorella sorokiniana* CY-1. The optimised parameters were ammonium concentration of 40 w/v% with volume ratio of 1:1 for ammonium sulphate and t-butanol; flotation air flowrate of 100 mL/min for 5 minutes; ultrasound pulse settings of 20 s ON/ 30 s OFF at pH of 6.5. The resultant protein separation efficiency, protein recovery, lipids recovery and carbohydrates recovery were 26.81±5.93%, 96.59±8.15%, 61.02±0.91% and 52.69±1.90%, respectively. The comparison study showed that U-LTF system gave higher yields than the control runs. A large-scale system (20×) observed similar yields. The recycling of phases was successful and extraction for multiple cycles is possible. The U-LTF system utilised in this study is a brilliant tool to utilise the full potential of microalgae with respect to its downstream processing. The biorefinery approach is vital to promote microalgae as a source of sustainable and renewable energy for the future.

CHAPTER 6

6. Preliminary *in vitro* evaluation of proteins extracted from *Chlorella vulgaris* coated on a gelatine-glutaraldehyde membrane and its wound healing application

6.1. Introduction

The largest organ of human body is skin, and an adult human being is estimated to have 3.6 kilograms of it str*etc*hing to 2 square metres. Wounds caused on human skin thus results in disruption of dermis and epidermis layer, which can expose the internal organs. Therefore, wound healing process is important to reduce the damage to skin. Wound healing is a complex process involving proliferation, migration, scar formation, inflammation and tissue remodelling [344]. In the event of extensive damage to the skin, it is important to accelerate the wound healing process to alle*via*te the immune system from the ROS build-up. Excessive generation of ROS results in disruption of DNA, oxidisation of lipids and proteins and eventually results in cell lysis which delays the wound healing process [345]. Therefore, studies have been conducted to utilise biomolecules from natural sources to accelerate wound healing. The most widely accepted method includes skin grafting by fish skin [346]. The bioactive compounds secreted by such natural sources are being studied for their anti-inflammatory, anti-cancer and anti-hypercholesterolemic properties [347].

Microalgae are considered a potential source of these bioactive compounds. Microalgae is a unicellular species with ability to secrete various bioactive molecules such as proteins, pigments, polysaccharides, vitamins and minerals which exhibit anti-viral, anti-cancer, antioxidant and anti-inflammatory properties [40]. *Spirulina platensis*, a green-blue cyanobacteria, has been extensively studied as wound healing material due to its potent anti-oxidative nature [348]. However, very few reports have been identified which studied *Chlorella vulgaris* for wound healing of human keratinocyte cells. Therefore, in

this study, *Chlorella vulgaris* was adsorbed on GGP membrane and its potential as a wound healing source was identified.

6.2. Materials and Methods

6.2.1. Materials

($(NH_4)_2SO_4$), t-butanol, Bradford's reagent, tris-HCl buffer and gelatine powder were purchased from R&M Chemicals (Malaysia). Cell culture grade trypsin, fetal bovine serum (FBS) was purchased from Sigma-Aldrich (USA). Minimum Essential Medium (MEM) was purchased from Thermo Fisher Scientific (USA). Phosphate-buffered saline (PBS) was prepared freshly with NaCl, KCl, KH_2PO_4 (Merck, Germany) and NaH_2PO_4 (Nacalai Tesque, Japan). Fluorescein diacetate (FDA), propidium iodide (PI), sodium bicarbonate and glutaraldehyde (GTA) solution were purchased from Sigma Aldrich, USA. Dimethyl sulfoxide (DMSO) was purchased from Merck, Germany. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Acros Organics, USA. Bio-safe Coomassie Stain, 2x Laemmli sample buffer, and 10 x Tris/Glycine/SDS were obtained from Bio-Rad Laboratories, USA. Dried *Chlorella vulgaris* powder were purchased from The Hive Bulk Foods Sdn. Bhd. (Kuala Lumpur, Malaysia). All the purchased solvents and chemicals were of analytical grade and were used without additional purification.

6.2.2. Microalgae protein extraction

The microalgae derived proteins were extracted by system U-LTF system as described in the previous study [328]. The freeze-dried microalgae biomass was extracted in the multiphase system and proteins were precipitated in the middle phase. This interfacial layer was pipetted out and washed with 0.1 M tris-HCl buffer. The extracted proteins were further purified by dialysis as described by Waghmare *et al.* [326]. The remaining salt in the extracted protein layer was removed by Hi-media dialysis membrane-50. The protein concentrate was added to the membrane and kept in 1 L of distilled water for 12

h. The resultant protein was pH adjusted to 5.0 and centrifuged for 10 min at 8000 g. This concentrate was transferred to a falcon tube and freeze dried for 24 h. The freeze-dried protein was used for further experimental runs.

6.2.3. Membrane preparation from gelatine-GTA

For membrane preparation, 100 mL of 5.0 w/v% gelatine solution was prepared and autoclaved. To the autoclaved gelatine solution, GTA solution in four different batches and stirred continuously for 2 h for hydro-gelling to achieve a concentration of 1.0 v/v%. Approximately 10 mL of gelatine-GTA mixture was transferred to each petri dish to create a thin layer of the membrane. These petri dishes were then freeze dried at -40 ºC for 36 h. The freeze-dried membranes were then immersed in 100, 70 and 50 % ethanol for 30 min each. The residual GTA blocks were blocked by further immersing these membranes in 100 mM glycine solution for 1 h. The membranes were then kept in a desiccator at room temperature for dehydration. Once all the water was removed, the dry membranes were cut by a hole puncher and sterilised in Biosafety cabinet (ESCO, Singapore) by placing each side under UV light for 15 min. The circular membranes were then stored in a sterilised petri-dish.

6.2.4. Cell culture and *in vitro* biological tests

6.2.4.1. HaCaT cell culture

HaCaT cells were thawed and cultured in T-75 tissue culture grade flask consisting of complete MEM supplemented with 5 % of FBS. The flasks were then placed in an incubator (Memmert, Germany) at 37.5 ºC with 5 % of CO_2. The flaks were routinely inspected for confluency and the complete MEM was replaced every 1-2 days. Once reached 70-80 % of confluency, the cells were subcultured. For cell subculture, the complete MEM was discarded, the flask was washed with PBS twice and 2 mL of 0.25 w/v% trypsin was added. This flask was incubated in the humidified incubator at 37.5 ºC with 5 % of CO_2 until the cells were detached from the surface.

6.2.4.2. Protein adsorption and cell seeding

Prior to cell-seeding, purified microalgal proteins were adsorbed on the gelatine-GTA membrane. The spherical membranes were placed in each well of a 96-well plate. Microalgae protein-PBS solutions of concentration of 100, 200, 500 and 1000 µg/mL were prepared in sterilised centrifuge tubes. A control group for pure protein (10P) with concentration of 1000 µg/mL was also prepared. Each membrane containing well was filled with 200 µL of specific protein concentration and was placed at room temperature for adsorption for 30 min. They were labelled as 1GGP, 2GGP, 5GGP, 10GGP. After 30 min, the remaining solution was pipetted out. Cells were further seeded on each of these wells with cell density of 2.5×10^5.

6.2.4.3. Cell adhesion test

The adhesion of cells to the membrane was observed *via* Zeiss Axio Observer for Biology fluorescence microscope (Zeiss, Germany) through cell staining. For this test, the cells were seeded in complete MEM and incubated for 4 h. The FDA stain was prepared by prepared by dissolving 1 mg of FDA in 1 mL of acetone. The concentration of 8 µg/mL required for the staining was attained by adding 8 µL of the prepared FDA solution to 1 mL of PBS. After seeding of cells on protein adsorbed membrane for 4 h, the complete MEM was discarded and replaced by 200 µL of the FDA stain for 5 min at room temperature in dark conditions. These wells were then washed twice with PBS and observed under the fluorescence microscope. The microscope was set at excitation wavelength of 535 nm and emission wavelength of 617 nm. The resulting images were captured with magnification of 5 x *via* Axiocam 503 mono camera with ZEN pro (Zeiss, Germany). The captured images of FDA-stained cells were utilised for semi-quantitative evaluation using ImageJ (National Institutes of Health, Bethesda, MD, USA).

6.2.4.4. Cell proliferation test

The HaCaT cells with density of 2.5 x 10^5 were seeded in 96 well plates with membranes of different protein concentrations. These wells were incubated for 1, 3 and 7 days at 37.5 ºC with 5 % of CO_2. A well with seeded cells without the membrane was used as a control and empty well was designated as a blank. For wells with 7 days assay, the old complete MEM was replaced at day 3. Once the incubation time of each assay was completed, the old complete MEM was replaced with a 200 µL of MEM and 20 µL of 5 mg/mL of MTT solution. The MTT-MEM solution wells were incubated for 4 h at 37 ºC. After 4 h, the MEM solution was discarded and 200 µL of DMSO was added to create purple formazan dye to signify the viable cells. After the purple crystals were completely dissolved, 100 µL of dye solution was transferred to a new separate well. The absorbance of the well was read at 570 nm by microplate reader (VersaMax, USA). The cell proliferation was calculated by following eq 6.1:

$$\text{Cell Proliferation } (\%) = \frac{OD\ of\ the\ protein{-}adsorped\ membrane}{OD\ of\ control} \times 100\% \qquad \text{(Eq 6.1)}$$

where OD means optical density.

6.2.4.5. Wound scratch assay

HaCaT cells were seeded on a 24 well plate (Orange Scientific, USA) until confluency was reached. After 24 h, the monolayer of cells was scratched by sterile p200 yellow pipette tip (Axygen Corning, USA) vertically to create a cell-free gap of 0.5 mm in each well. In a separate 96 well plate, protein adsorption on each membrane at different concentrations was carried out by immersing the membrane in 200 µL of protein-PBS solution for 30 min at room temperature. After 30 min, the remaining solution was discarded. To these wells, 200 µL of complete MEM was added and incubated at 37 ºC for 20 min. This complete MEM was then transferred to corresponding scratched 24 well plate. The well plate with only complete MEM was assigned as a control. The well plate was captured after wound scratch and after 24 h to observe the cell growth. The gap

between each scratch was quantified and percentage wound closure was calculated by eq 6.2:

$$Wound\ closure\ (\%) = \frac{Gap\ at\ 0h\ (mm) - Gap\ at\ 24h\ (mm)}{Gap\ at\ 0h} \times 100\% \qquad \text{(Eq 6.2)}$$

6.3. Results and Discussion

6.3.1. Cell viability and proliferation

The cell viability of the seeded HaCaT cells on the GGP membrane was tested by MTT assay and results for 1, 3 and 7 days are depicted in Figure 6.1. The results are expressed as viable cells compared to positive control (only cells). The highest proliferation of 76.75±0.68 % for 1 day was observed at 10P. This was followed by 10GGP and 5GGP with cell viability of 48.23±0.58 % and 40.93±0.52 %. The lowest proliferation was observed with 1GGP, 2GGP and GG membrane at 15.75±0.08 %, 16.75±0.06 % and 17.21±0.04 %, respectively. The MTT results after 3 days were slightly better for 5GGP with cell viability of 44.75±0.67 %. The cell viability of 1GGP and 2GGP were still below 25 %. Although, the viability of 10P decreased from 1 to 3 days and remained constant until 7 days at around 50 %. The lowest cell viability was noted for control group consisting of membrane only ranging between 17-20 %.

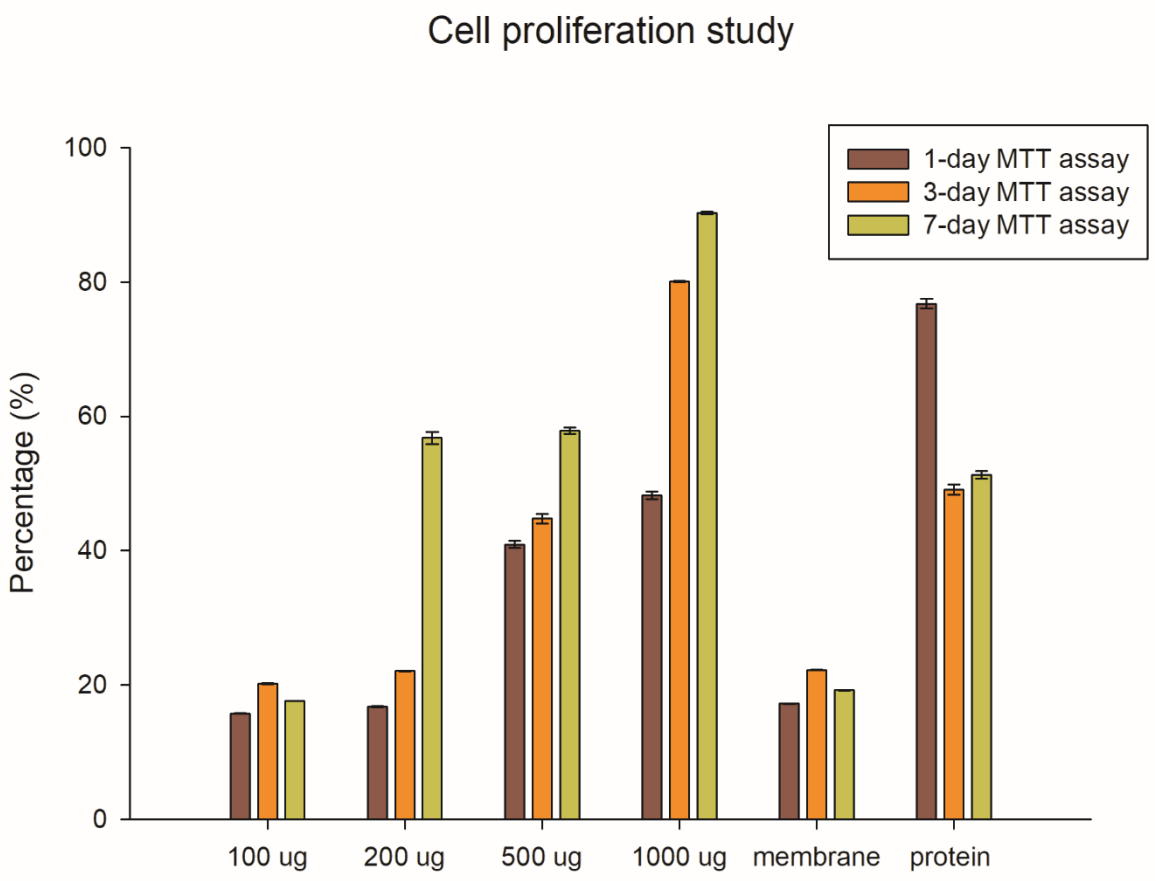


Figure 6.1 Cell proliferation of HaCaT cells detected by MTT assay on GGP membrane for 1, 3 and 7 days.

After 7 days, 2GGP, 5GGP and 10GGP exhibited proliferation above 50 % with 10GGP being highest at 90.26±0.22 %. Syarina *et al.* experimented the cytotoxicity effect of *Spirulina* sp. extracts on human primary dermal fibroblast (HDF) cells and observed a similar result as this experiment. The study concluded that 300 µg/mL of *Spirulina* aqueous extract achieved cell proliferation above 90 % [349]. The consistent low cell viability of pure membrane group can be explained by high cytotoxicity displayed by GTA. Yang *et al.* conducted experiment on biocompatibility of gelatine with various crosslinking materials and concluded that scaffolds with GTA concentrations higher than 0.5 v/v% with gelatine are toxic for adipose-derived stromal stem cells [350]. From Figure 6.1, an increasing trend is observed for cell proliferation from 1 to 7 days. Similar observations

were made by Jung *et al.* in a cytotoxicity study conducted by introducing *Spirulina*-PCL nanofiber onto rat dermal fibroblast. The study concluded that MTT assay recorded higher cell viability from 1-5 days and hypothesised that microalgae in the culture media is responsible for a higher cell proliferation [351]. The statistical analysis conducted reveals that the differences in the data was significant as denoted by p-value <0.05. Overall, 10GGP membrane showcased cytocompatibility and supported the *in vitro* growth of keratinocyte cells.

6.3.2. Cell adhesion test

The cell adhesion test was observed by the green FDA-stained cells on the membrane. Figure 6.2 depicts the stained cells on all the GGP membrane systems and control wells. The high fluorescence intensity of 1GGP and 10GGP corresponds to high number of viable HaCaT cells on the membrane. This suggests that the microalgae proteins are biocompatible with the keratinocyte cells and are not harmful to human skin. It is well known that gelatine membrane consists of numerous sites for cell binding and growth, although is not stable in liquid media and requires GTA crosslinking to increase the stability [352].

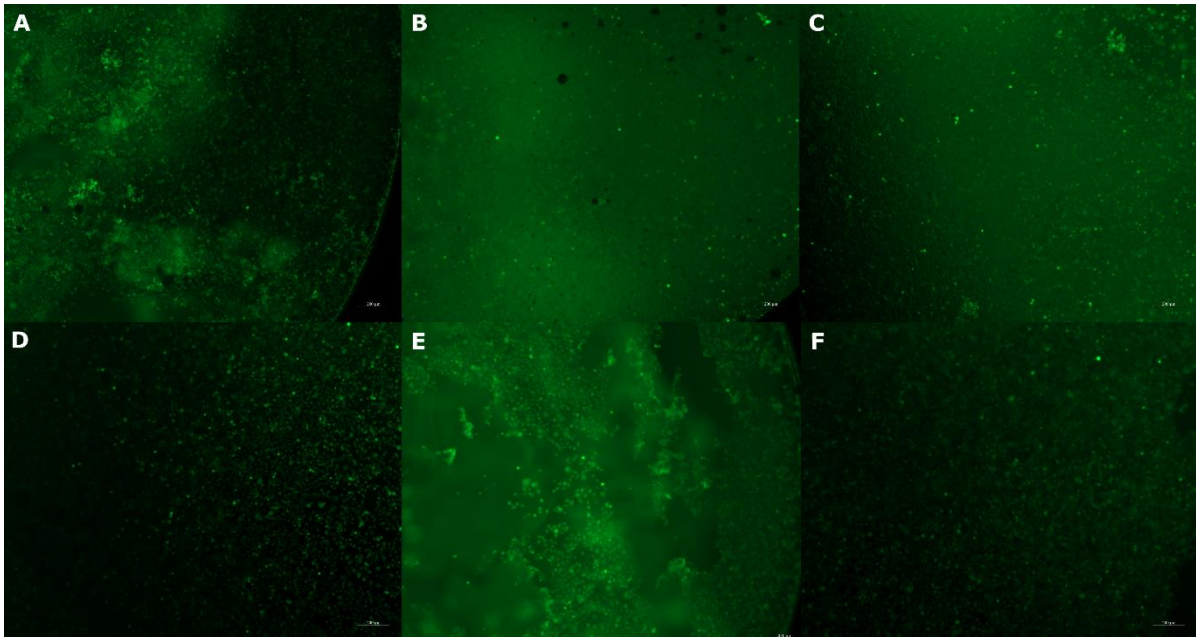


Figure 6.2 Observation of GGP adhered to HaCaT cells by fluorescent microscope: A) 1GGP, B) 2GGP, C) 5GGP, D) 10GGP, E) membrane and F) control cells.

The semi-quantitative analysis of the cell count found that the membrane and 5GGP adhered to the least number of cells (Figure 6.3). The lowest cell count of 1249±352 was observed for pure membrane system, whereas 10GGP recorded highest viable stained cell number of 3432±443. The low count was observed due to potential cytotoxicity associated with high concentration of GTA [353]. Studies have indicated that high degrees of crosslinking are observed between gelatine and GTA biopolymers thus enhancing the cell adhesion [354–356]. This can be observed with the high number of cell count by fluorescence spectrophotometer. Adilet et al., conducted a study on cell viability of fish scale protein derived gelatine nanofibrous scaffolds and concluded that increase in concentration of fish scale gelatine resulted in higher number of viable cells [357]. Hassan et al., conducted a similar study utilizing fish biowaste gelatine and phosphate glass fibres also concluded that increase in gelatine concentration resulted in increase in viable number of cells [358]. A similar trend was observed in this study with higher protein concentration resulted in higher number of viable cells. The statistical

analysis conducted reveals that the differences in the data was significant as denoted by p-value <0.05.

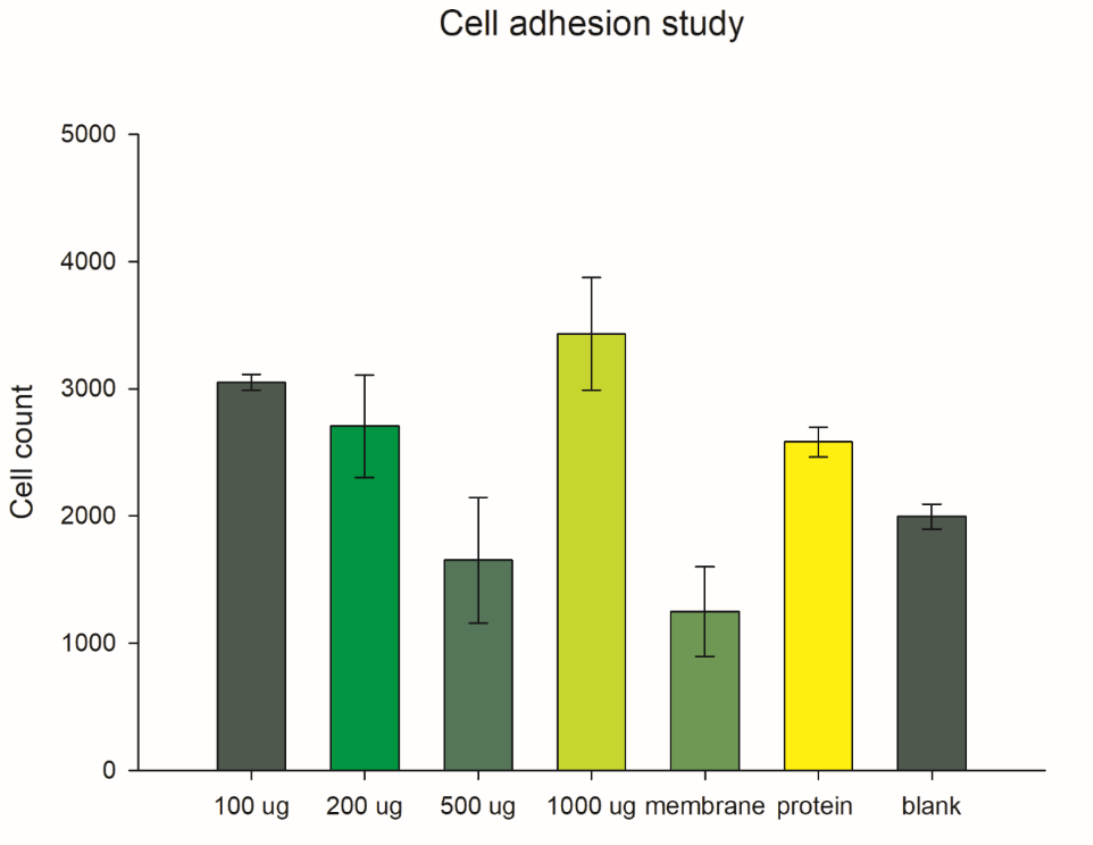


Figure 6.3 Quantification of FDA-stained viable keratinocyte cells on GGP membrane and control studies.

6.3.3. *In vitro* wound healing

The cell migration effect of microalgae protein adsorbed on gelatine-GTA membrane was observed in this assay. Figure 6.4 illustrates the wound closure of control (blank), membrane and the best result (10GGP) of the HaCaT cells at 0, 12 and 24 h after the scratch. The cell migration as a measure of distance of wound closure is depicted in Figure 6.5. At 12 h, highest wound closure of 39.97±0.15 % was observed for 10GGP followed by 31.93±0.4 % for 5GGP. The pure membrane system recorded lowest wound closure of 10.23±0.25 %. The highest wound closure% after 24 h was observed for 10GGP (82.10±1.65 %) followed by 5GGP (77.97±1.45 %). The lowest wound closure%

after 24 h of 40.5±1.04 % was observed for pure membrane system. The pure protein system had higher wound closure% compared to blank, thus proving that microalgae protein contributes towards accelerated wound closure. Bari *et al.* studied the effectiveness of microsphere based on *Chlorella vulgaris* and *Spirulina platensis* with silk sericin on Human Fibroblast cells and concluded that total wound closure was observed at 72 h [359].

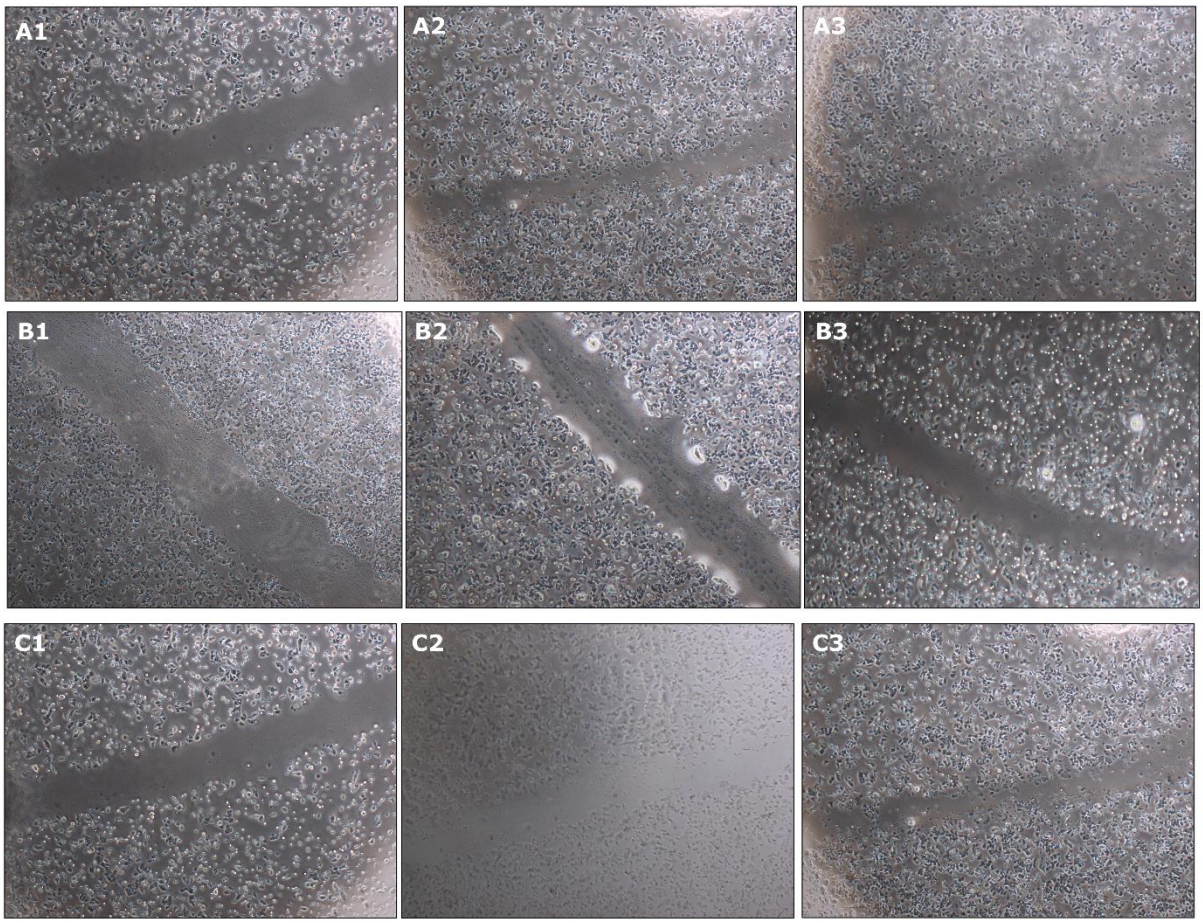


Figure 6.4 *In vitro* wound healing effect by representative images of GGP membrane on HaCaT cells: A) 10GGP, B)membrane, C) control cells. While 1, 2 and 3 represent 0, 12 and 24 h.

The increased wound closure for higher protein concentration was due to a pigment, β-carotene, present in the *Chlorella vulgaris* biomass which increases cell defense and enhances tissue integrity. Additionally, Machmud *et al.* hypothesised that *Chlorella*

growth factor which constitutes of numerous amino acids, minerals, vitamins and nucleic acids assist in keratinocytes regeneration process [360]. Zailan *et al.* confirmed that *Chlorella vulgaris* dressing was able to accelerate wound healing in rats with minimized formation of scar tissues [361]. According to Veeraperumal *et al.*, cell proliferation and migration is dependent on numerous processes and is divided into different phases like G1, G2, M and S phase. The S phase corresponds to DNA synthesis and is an indicator of cell migration activity. The G1 and G2 phases correspond to pre and post DNA synthesis, respectively. The study conducted by Veeraperumal *et al.* on wound healing of keratinocytes by polysaccharides from *Gracilaria lemaneiformis* concluded that the purified polysaccharide fraction regulated the cell migration by enhancing DNA content during S phase [362]. Liu *et al.* prepared *Spirulina* extracts (SPCP) and tested its wound healing properties on human dermal fibroblast cells (CCD-986sk) and concluded that SPCP promotes cell migration and proliferation [363]. The authors hypothesised that according to the cell cycle analysis, SPCP assisted the cells in entering S phase from G1 phase. The statistical analysis conducted reveals that the differences in the data was significant as denoted by p-value <0.05.

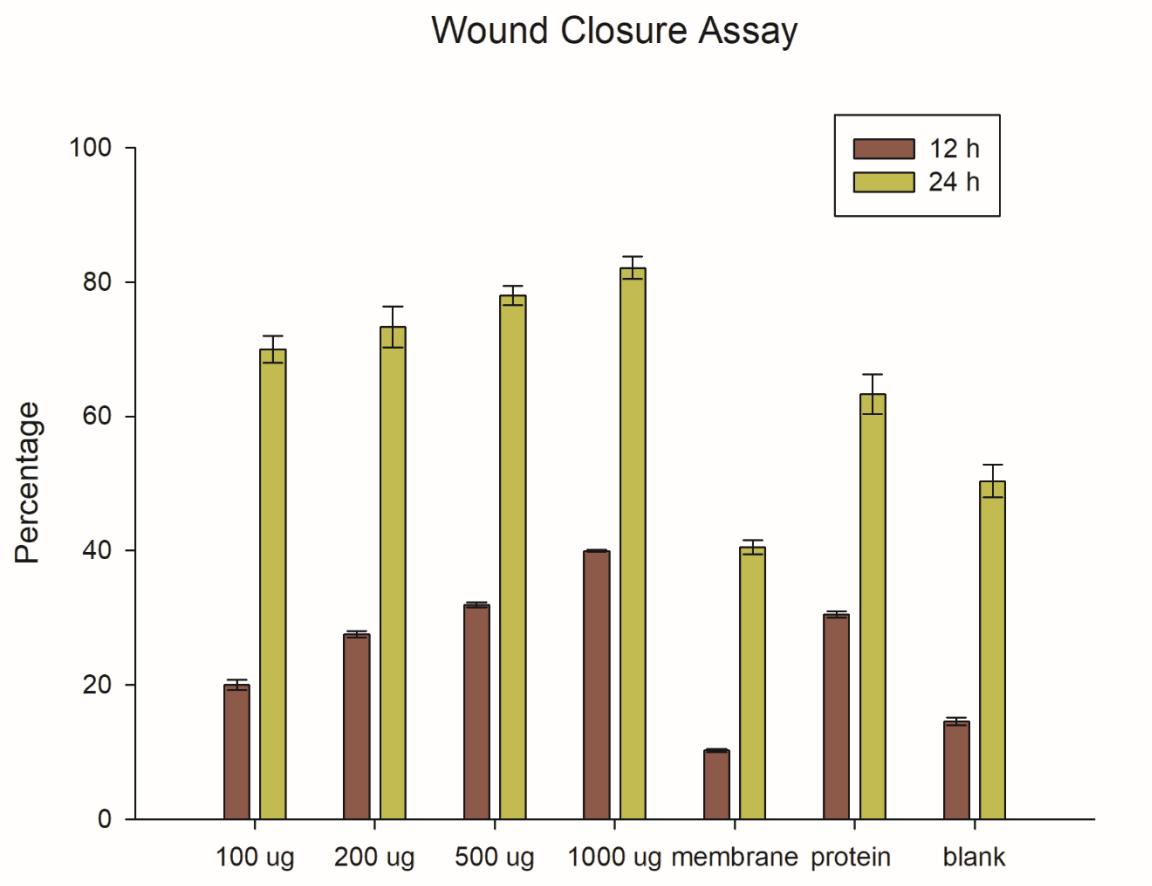


Figure 6.5 Wound closure percentage of HaCaT cells after treatment with GGP membrane at 12 and 24 h.

The biocompatibility tests conducted resulted in a successful cell migration of HaCaT cells with microalgae protein adsorbed gelatine-GTA membrane. However, for future studies, analytical studies can be performed to confirm the morphological properties of the membrane.

6.4. Conclusions

The final study of this thesis is based on purified proteins extracted from *Chlorella vulgaris* by U-LTF system. The proteins were adsorbed on GGP membranes and this system was utilised to examine the cell proliferation, adhesion and migration on HaCaT cells. This study was successful in establishing the wound healing properties of *Chlorella vulgaris* derived proteins on human keratinocyte cells. The best performing system was

10GGP with cell proliferation of 90.26±0.22 % after 7 days. This set-up also resulted in the highest viable adherence of cells with estimated cell count of 3432±443 and the highest wound closure% of 82.10±1.65 % after 24 h. The wound healing application of purified proteins from microalgae by U-LTF system thus demonstrates the implementation of microalgae biorefinery in the industry. Thus, 10 GGP system can be potentially utilised as a wound healing material in future.

CHAPTER 7

7. Conclusions and future research

The potential of microalgal biomass to produce high-value bioactive components enables it as a promising raw material for bioprocessing. This thesis focused on obtaining various products from microalgae *via* biorefinery approach. The lipids extracted can be utilised as health supplements in form of PUFA in addition to biodiesel production, while proteins and carbohydrates can be used in diets and in fermentation industry, respectively. Various technologies are being investigated for obtaining the components with high production rate, ease of operation, higher yield and lower cost. However, these processes are still in the infant stage. This thesis explored extraction of proteins by sugaring-out method and potential microalgae biorefinery by U-LTF system. As the ACN is toxic for food-consumption, less toxic solvents were considered for the next 2 studies. The utilization of t-butanol resulted in high extraction of protein, lipid and carbohydrate from microalgae. From the results obtained, highest extraction of protein was observed followed by lipid and carbohydrates. Therefore, it can be concluded that the biorefinery should be based on protein extraction for either feed or wound-healing application presented here. This can be followed by biofuel production through lipid and carbohydrate.

7.1. Optimisation of protein extraction from *Chlorella vulgaris via* novel sugaring-out assisted LBEF system

In this experiment, microalgae specie of *Chlorella vulgaris* FSP-E was utilised for protein extraction *via* simple sugaring-out assisted Liquid Biphasic Electric Flotation system (LBEF). The external electric force provided to the two-phase system assists in disruption of rigid microalgae cell wall and releases the contents of microalgae cell. This experiment manipulates various parameters to optimise the set-up. The LBEF set-up is compared with a control Liquid Biphasic Flotation experiment without the electric field supply. The

optimised separation efficiency of the LBEF system was 73.99±0.74 % and protein recovery of 69.66±0.86 % compared with Liquid Biphasic Flotation, the separation efficiency was 61.58±0.36 % and protein recovery was 48.78±0.48 %. The separation efficiency and protein recovery for 5× time scaled-up system was observed at 52.871±1.23 % and 73.29±0.70 %. The results confirmed that objectives set at the beginning of the study were achieved. The integration of simultaneous cell-disruption and protein extraction ensures high yield of protein from microalgae.

7.2. Biorefinery of *Chlorella sorokiniana* using U-LTF system

The aim of this experiment was to study ultrasonication-assisted Liquid Triphasic Flotation (U-LTF) System to obtain lipid and protein from microalgae *Chlorella sorokiniana* in a single step as a novel process. In the current study, a novel ultrasonication-assisted Liquid Triphasic Flotation (LTF) System was utilised to obtain lipid and protein from microalgae *Chlorella sorokiniana* in a single step. The highest protein recovery of 97.43±1.67 % and lipid recovery of 69.50±0.54 % were obtained. The corresponding parameters were microalgal biomass loading of 0.5 w/v%, $(NH_4)_2SO_4$ concentration of 40 w/v%, volume ratio of 1:1.5 (salt:alcohol), ultrasonication pulse mode of 20 s ON/20s OFF at 20 % amplitude for 5 min, flotation air flowrate of 100 mL/min. Additionally, recycling of alcohol phase to study the circular nature of proposed biorefinery was investigated. The results confirmed that objectives set at the beginning of the study were achieved.

7.3. Liquid triphasic systems as a sustainable downstream processing of *Chlorella* biorefinery for potential biofuels and feed production

This study focused on optimisation of different parameters of ultrasonication-assisted LTF system (U-LTF) to extract proteins, lipids and carbohydrates from *Chlorella sorokiniana* CY-1. The optimised parameters were ammonium concentration of 40 w/v% with volume ratio of 1:1 for $(NH_4)_2SO_4$:t-butanol; flotation air flowrate of 100 mL/min

for 5 min; ultrasound pulse settings of 20 s ON/ 30s OFF at pH of 6.5. The resultant protein separation efficiency, protein recovery, lipids recovery and carbohydrates recovery were 26.81±5.93 %, 96.59±8.15 %, 61.02±0.91 % and 52.69±1.90 % respectively. The comparison study showed that U-LTF system gave higher yields than the control runs. A large-scale system (20 ×) observed similar yields. The recycling of phases was successful and extraction for multiple cycles is possible. The results confirmed that objectives set at the beginning of the study were achieved.

7.4. Preliminary *in vitro* evaluation of proteins from *Chlorella vulgaris* coated on gelatine-glutaraldehyde membrane and its wound healing application

In this study, proteins extracted from *Chlorella vulgaris* were adsorbed on a GGP membrane. The biocompatibility of this protein-adsorbed membrane was examined on human keratinocyte cells. The cell proliferation (MTT assay), cell adhesion (FDA staining) and wound healing test were conducted. The best performing system was 10GGP with cell proliferation of 90.26±0.22 % after 7 days. This set-up also resulted in highest viable adherence of cells with estimated cell count of 3432±443 and highest wound closure% of 82.10±1.65 % after 24 h. The study successfully demonstrated the effectiveness of microalgae proteins on wound healing of HaCaT skin cells. The results confirmed that objectives set at the beginning of the study were achieved.

7.5. Future research

In this thesis, multiple component extraction from microalgae biomass was examined. The first study explored liquid biphasic flotation method coupled with electrolysis. The main limiting factors of this study was the low voltage of electrolysis. Future work needs to explore high voltage electricity such as High Voltage Electric Discharge (HVED) or Pulsed Electric Field (PEF) as a cell-disruption method. Moreover, ACN is considered as a highly toxic, flammable solvent and resultant purified proteins might not be

biocompatible. The biorefinery of *Chlorella* by U-LTF systems demonstrated high recovery of multiple components by single system and were successful in displaying the potential of microalgal biomass. The systems performed were in laboratory scale, including the large-scale system which had working volume of 1.5-2.0 L. Therefore, pilot scale plants need to be set-up to explore the efficiency of LTF system. A successful pilot plant will increase the confidence of investors in further commercialization of this system. Furthermore, purification of one component (proteins) was conducted in the last study. A biorefinery work with extraction, purification and end products of all the biomolecules ready for commercial usage is the end goal. Thus, future studies can focus on purification of all the three components and application. This thesis, however, boosts the current research in a pathway to successfully achieve biorefinery of microalgae. LCA and economic assessment of current large-scale processes with single product or two products system from microalgae deem it infeasible. The possibility of producing multiple bioactive components from a single microalgae strain has attracted the attention of researchers to optimise and streamline the material and energy balances. However, with current downstream processing techniques, multiple product extraction is not economical as the whole biorefinery creates more emissions. This issue can be tackled by research and development of simple and cheap downstream processing techniques. Therefore, this thesis focused on microalgae biorefinery as a fundamental step before commercialisation. To conclude, microalgae biorefinery by multiphase systems such as U-LTF conducted in this thesis paves a way to solve shortages of fossil fuels and feed experienced by mankind due to increasing global population.

References

[1] Know Your World: Facts About World Hunger & Poverty, (n.d.). http://www.thp.org/knowledge-center/know-your-world-facts-about-hunger-poverty/.

[2] FAO - News Article: World's future food security "in jeopardy" due to multiple challenges, report warns, (n.d.). http://www.fao.org/news/story/en/item/471169/icode/.

[3] J.J. Milledge, Commercial application of microalgae other than as biofuels: a brief review, Rev. Environ. Sci. Biotechnol. (2011). doi:10.1007/s11157-010-9214-7.

[4] E. Christaki, P. Florou-Paneri, E. Bonos, Microalgae: A novel ingredient in nutrition, Int. J. Food Sci. Nutr. 62 (2011) 794–799. doi:10.3109/09637486.2011.582460.

[5] H. Wolkers, M. Barbosa, D. Kleinegris, R. Bosma, R.H. Wijffels, Microalgae: the green gold of the future?, Wageningen, 2011. www.groenegrondstoffen.nl (accessed November 27, 2018).

[6] S. Bleakley, M. Hayes, Algal Proteins: Extraction, Application, and Challenges Concerning Production, Foods. 6 (2017) 1–34. doi:10.3390/foods6050033.

[7] M.M. van Krimpen, P. Bikker, I.M. van der Meer, C.M.C. van der Peet-Schwering, J.M. Vereijken, Cultivation, processing and nutritional aspects for pigs and poultry of European protein sources as alternatives for imported soybean products, Wageningen, 2013. http://www.livestockresearch.wur.nl (accessed November 26, 2018).

[8] J.S. Wallace, Increasing agricultural water use efficiency to meet future food production, 2000. https://pdfs.semanticscholar.org/cdcb/d4447f604b2e1c95c513edab6d0a004224d9.pdf (accessed November 26, 2018).

[9] D. Pimentel, M. Pimentel, Sustainability of meat-based and plant-based diets and

the environment, 2003. https://academic.oup.com/ajcn/article-abstract/78/3/660S/4690010 (accessed November 26, 2018).

[10] P. Sampath-Wiley, C.D. Neefus, L.S. Jahnke, Seasonal effects of sun exposure and emersion on intertidal seaweed physiology: Fluctuations in antioxidant contents, photosynthetic pigments and photosynthetic efficiency in the red alga Porphyra umbilicalis Kützing (Rhodophyta, Bangiales), (2008). doi:10.1016/j.jembe.2008.05.001.

[11] P. Spolaore, C. Joannis-Cassan, E. Duran, A. Isambert, Commercial applications of microalgae, J. Biosci. Bioeng. 101 (2006) 87–96. doi:10.1263/jbb.101.87.

[12] L. Gouveia, A.P. Batista, I. Sousa, A. Raymundo, N.M. Bandarra, Microalgae in Novel Food Product, in: Food Chem. Res. Dev., Nova Science Publishers, Inc., 2008: pp. 1–37.

[13] T.M. Mata, A.A. nio Martins, N.S. Caetano, Microalgae for biodiesel production and other applications: A review, Renew. Sustain. Energy Rev. 14 (2009) 217–232. doi:10.1016/j.rser.2009.07.020.

[14] T. Bruton, A Review of the Potential of Marine Algae as a Source of Biofuel in Ireland, Ireland, 2009.

[15] T. Cai, S.Y. Park, Y. Li, Nutrient recovery from wastewater streams by microalgae: Status and prospects, Renew. Sustain. Energy Rev. 19 (2013) 360–369. doi:10.1016/j.rser.2012.11.030.

[16] Y. Li, M. Horsman, B. Wang, N. Wu, C.Q. Lan, Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans, Appl. Microbiol. Biotechnol. 81 (2008) 629–636. doi:10.1007/s00253-008-1681-1.

[17] Q. Hu, M. Sommerfeld, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert, A. Darzins, Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances, Plant J. 54 (2008) 621–639. doi:10.1111/j.1365-313X.2008.03492.x.

[18] R.S. Gour, M. Bairagi, V.K. Garlapati, A. Kant, Enhanced microalgal lipid production with media engineering of potassium nitrate as a nitrogen source, Bioengineered. 9 (2018) 98–107. doi:10.1080/21655979.2017.1316440.

[19] J. Zhang, X. Fang, X.-L. Zhu, Y. Li, H.-P. Xu, B.-F. Zhao, L. Chen, X.-D. Zhang, Microbial lipid production by the oleaginous yeast Cryptococcus curvatus O3 grown in fed-batch culture, Biomass and Bioenergy. 35 (2011) 1906–1911. doi:10.1016/J.BIOMBIOE.2011.01.024.

[20] S. Fakas, S. Papanikolaou, A. Batsos, M. Galiotou-Panayotou, A. Mallouchos, G. Aggelis, Evaluating renewable carbon sources as substrates for single cell oil production by Cunninghamella echinulata and Mortierella isabellina, Biomass and Bioenergy. 33 (2009) 573–580. doi:10.1016/J.BIOMBIOE.2008.09.006.

[21] H.S. Kwak, J.Y.H. Kim, H.M. Woo, E. Jin, B.K. Min, S.J. Sim, Synergistic effect of multiple stress conditions for improving microalgal lipid production, Algal Res. 19 (2016) 215–224. doi:10.1016/J.ALGAL.2016.09.003.

[22] A. Abu-Shamleh, Y.S.H. Najjar, Optimization of mechanical harvesting of microalgae by centrifugation for biofuels production, Biomass and Bioenergy. 143 (2020) 105877. doi:https://doi.org/10.1016/j.biombioe.2020.105877.

[23] A. Ortiz, M.J. García-Galán, J. García, R. Díez-Montero, Optimization and operation of a demonstrative full scale microalgae harvesting unit based on coagulation, flocculation and sedimentation, Sep. Purif. Technol. 259 (2021) 118171. doi:https://doi.org/10.1016/j.seppur.2020.118171.

[24] E.T. Chua, E. Eltanahy, H. Jung, M. Uy, S.R. Thomas-Hall, P.M. Schenk, Efficient Harvesting of Nannochloropsis Microalgae via Optimized Chitosan-Mediated Flocculation, Glob. Challenges. 3 (2019) 1800038. doi:https://doi.org/10.1002/gch2.201800038.

[25] L. de Souza Leite, P.R. dos Santos, L.A. Daniel, Microalgae harvesting from

wastewater by pH modulation and flotation: Assessing and optimizing operational parameters, J. Environ. Manage. 254 (2020) 109825. doi:https://doi.org/10.1016/j.jenvman.2019.109825.

[26] A. Pandey, R. Shah, P. Yadav, R. Verma, S. Srivastava, Harvesting of freshwater microalgae Scenedesmus sp. by electro-coagulation-flocculation for biofuel production: effects on spent medium recycling and lipid extraction, Environ. Sci. Pollut. Res. 27 (2020) 3497–3507. doi:10.1007/s11356-019-06897-y.

[27] M. Alhattab, M.S.-L. Brooks, Optimization of Chlorella saccharophila harvesting by surfactant-aided dispersed air flotation for biodiesel production processes, Biomass and Bioenergy. 134 (2020) 105472. doi:https://doi.org/10.1016/j.biombioe.2020.105472.

[28] S. Wahidin, A. Idris, N.M. Yusof, N.H.H. Kamis, S.R.M. Shaleh, Optimization of the ionic liquid-microwave assisted one-step biodiesel production process from wet microalgal biomass, Energy Convers. Manag. 171 (2018) 1397–1404. doi:https://doi.org/10.1016/j.enconman.2018.06.083.

[29] M.A. Rahman, M.A. Aziz, R.A. Al-khulaidi, N. Sakib, M. Islam, Biodiesel production from microalgae Spirulina maxima by two step process: Optimization of process variable, J. Radiat. Res. Appl. Sci. 10 (2017) 140–147. doi:10.1016/j.jrras.2017.02.004.

[30] A. San Pedro, C. V González-López, F.G. Acién, E. Molina-Grima, Marine microalgae selection and culture conditions optimization for biodiesel production, Bioresour. Technol. 134 (2013) 353–361. doi:https://doi.org/10.1016/j.biortech.2013.02.032.

[31] A. Barka, C. Blecker, Microalgae as a potential source of single-cell proteins. A review, Biotechnol. Agron. Soc. Env. 20 (2016) 427–436. https://popups.uliege.be/1780-4507/index.php?id=16755&file=1&pid=13132

(accessed November 21, 2018).

[32] K. Moorhead, B. Capelli, G.R. Cysewski, SPIRULINA Nature's Superfood, 3rd ed., Hawaii, 2011.

[33] A.K. Koyande, P.-L. Show, R. Guo, B. Tang, C. Ogino, J.-S. Chang, Bio-processing of algal bio-refinery: a review on current advances and future perspectives, Bioengineered. 10 (2019) 574–592. doi:10.1080/21655979.2019.1679697.

[34] T.L. Chacón-Lee, G.E. González-Mariño, Microalgae for "Healthy" Foods—Possibilities and Challenges, Compr. Rev. Food Sci. Food Saf. 9 (2010) 655–675. doi:10.1111/j.1541-4337.2010.00132.x.

[35] M. Muto, D. Nojima, L. Yue, H. Kanehara, H. Naruse, A. Ujiro, T. Yoshino, T. Matsunaga, T. Tanaka, Potential of water surface-floating microalgae for biodiesel production: Floating-biomass and lipid productivities, J. Biosci. Bioeng. 123 (2017) 314–318. doi:10.1016/J.JBIOSC.2016.09.015.

[36] D.-T. Tran, C.-L. Chen, Effect of solvents and oil content on direct transesterification of wet oil-bearing microalgal biomass of Chlorella vulgaris ESP-31 for biodiesel synthesis using immobilized lipase as the biocatalyst, Bioresour. Technol. 135 (2013) 213–221. doi:10.1016/J.BIORTECH.2012.09.101.

[37] W.Y. Cheah, P.L. Show, T.C. Ling, J.C. Juan, Biosequestration of atmospheric CO2 and flue gas-containing CO2 by microalgae, Bioresour. Technol. 184 (2015) 190–201. doi:10.1016/J.BIORTECH.2014.11.026.

[38] S. Sen Roy, R. Pal, Microalgae in Aquaculture: A Review with Special References to Nutritional Value and Fish Dietetics, Proc. Zool. Soc. 68 (2015) 1–8. doi:10.1007/s12595-013-0089-9.

[39] C.V. Viegas, I. Hachemi, P. Mäki-Arvela, A. Smeds, A. Aho, S.P. Freitas, C.M. da Silva Gorgônio, G. Carbonetti, M. Peurla, J. Paranko, N. Kumar, D.A.G. Aranda, D.Y. Murzin, Algal products beyond lipids: Comprehensive characterization of different

products in direct saponification of green alga Chlorella sp., Algal Res. 11 (2015) 156–164. doi:10.1016/J.ALGAL.2015.06.014.

[40] A.K. Koyande, K.W. Chew, K. Rambabu, Y. Tao, D.-T. Chu, P.-L. Show, Microalgae: A potential alternative to health supplementation for humans, Food Sci. Hum. Wellness. 8 (2019) 16–24. doi:10.1016/j.fshw.2019.03.001.

[41] E. Manirafasha, T. Ndikubwimana, X. Zeng, Y. Lu, K. Jing, Phycobiliprotein: Potential microalgae derived pharmaceutical and biological reagent, Biochem. Eng. J. 109 (2016) 282–296. doi:10.1016/J.BEJ.2016.01.025.

[42] A.V. Ursu, A. Marcati, T. Sayd, V. Sante-Lhoutellier, G. Djelveh, P. Michaud, Extraction, fractionation and functional properties of proteins from the microalgae Chlorella vulgaris, Bioresour. Technol. 157 (2014) 134–139. doi:10.1016/j.biortech.2014.01.071.

[43] M.M. Phukan, R.S. Chutia, B.K. Konwar, R. Kataki, Microalgae Chlorella as a potential bio-energy feedstock, Appl. Energy. 88 (2011) 3307–3312. doi:10.1016/j.apenergy.2010.11.026.

[44] R. Sankaran, P.L. Show, Y.S. Cheng, Y. Tao, X. Ao, T.D.P. Nguyen, D. Van Quyen, Integration Process for Protein Extraction from Microalgae Using Liquid Biphasic Electric Flotation (LBEF) System, Mol. Biotechnol. 60 (2018) 749–761. doi:10.1007/s12033-018-0111-6.

[45] D. Pimentel, A. Marklein, M.A. Toth, M.N. Karpoff, G.S. Paul, R. McCormack, J. Kyriazis, T. Krueger, Food Versus Biofuels: Environmental and Economic Costs, Hum. Ecol. 37 (2009) 1. doi:10.1007/s10745-009-9215-8.

[46] I. Hariskos, C. Posten, Biorefinery of microalgae – opportunities and constraints for different production scenarios, Biotechnol. J. 9 (2014) 739–752. doi:10.1002/biot.201300142.

[47] J. Hill, E. Nelson, D. Tilman, S. Polasky, D. Tiffany, Environmental, economic, and

energetic costs and benefits of biodiesel and ethanol biofuels, Proc. Natl. Acad. Sci. 103 (2006) 11206–11210. doi:10.1073/pnas.0604600103.

[48] A. Ajanovic, Biofuels versus food production: Does biofuels production increase food prices?, Energy. 36 (2011) 2070–2076. doi:10.1016/J.ENERGY.2010.05.019.

[49] C. Abels, F. Carstensen, M. Wessling, Membrane processes in biorefinery applications, J. Memb. Sci. 444 (2013) 285–317. doi:10.1016/J.MEMSCI.2013.05.030.

[50] M.O.S. Dias, T.L. Junqueira, O. Cavalett, M.P. Cunha, C.D.F. Jesus, P.E. Mantelatto, C.E.V. Rossell, R. Maciel Filho, A. Bonomi, Cogeneration in integrated first and second generation ethanol from sugarcane, Chem. Eng. Res. Des. 91 (2013) 1411–1417. doi:10.1016/J.CHERD.2013.05.009.

[51] V. Menon, M. Rao, Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept, Prog. Energy Combust. Sci. 38 (2012) 522–550. doi:10.1016/J.PECS.2012.02.002.

[52] R.A. Lee, J.-M. Lavoie, From first- to third-generation biofuels: Challenges of producing a commodity from a biomass of increasing complexity, Anim. Front. 3 (2013) 6–11. doi:10.2527/af.2013-0010.

[53] M.-K. Ji, R.A.I. Abou-Shanab, S.-H. Kim, E.-S. Salama, S.-H. Lee, A.N. Kabra, Y.-S. Lee, S. Hong, B.-H. Jeon, Cultivation of microalgae species in tertiary municipal wastewater supplemented with CO2 for nutrient removal and biomass production, Ecol. Eng. 58 (2013) 142–148. doi:10.1016/J.ECOLENG.2013.06.020.

[54] R. Dillschneider, C. Steinweg, R. Rosello-Sastre, C. Posten, Biofuels from microalgae: Photoconversion efficiency during lipid accumulation, Bioresour. Technol. 142 (2013) 647–654. doi:10.1016/J.BIORTECH.2013.05.088.

[55] L. Brennan, P. Owende, Biofuels from microalgae — A review of technologies for production , processing , and extractions of biofuels and co-products, Renew.

Sustain. Energy Rev. 14 (2010) 557–577. doi:10.1016/j.rser.2009.10.009.

[56] F. Gabriel Acien Fernandez, C. V. González-López, J.M. Fernández Sevilla, E. Molina Grima, Conversion of CO 2 into biomass by microalgae: How realistic a contribution may it be to significant CO 2 removal?, Appl. Microbiol. Biotechnol. 96 (2012) 577–586. doi:10.1007/s00253-012-4362-z.

[57] T. Suganya, M. Varman, H.H. Masjuki, S. Renganathan, Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach, Renew. Sustain. Energy Rev. 55 (2016) 909–941. doi:10.1016/J.RSER.2015.11.026.

[58] K.Y. Show, Y. Yan, M. Ling, G. Ye, T. Li, D.J. Lee, Hydrogen production from algal biomass – Advances, challenges and prospects, Bioresour. Technol. 257 (2018) 290–300. doi:10.1016/j.biortech.2018.02.105.

[59] A. Melis, L. Zhang, M. Forestier, M.L. Ghirardi, M. Seibert, Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga Chlamydomonas reinhardtii., Plant Physiol. 122 (2000) 127–36.
http://www.ncbi.nlm.nih.gov/pubmed/10631256%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC58851.

[60] A. Raheem, W.A.K.G. Wan Azlina, Y.H. Taufiq Yap, M.K. Danquah, R. Harun, Optimization of the microalgae Chlorella vulgaris for syngas production using central composite design, RSC Adv. 5 (2015) 71805–71815. doi:10.1039/c5ra10503j.

[61] H.B. Goyal, D. Seal, R.C. Saxena, Bio-fuels from thermochemical conversion of renewable resources: A review, Renew. Sustain. Energy Rev. 12 (2008) 504–517. doi:10.1016/J.RSER.2006.07.014.

[62] K.. Kadam, Environmental implications of power generation via coal-microalgae

cofiring, Energy. 27 (2002) 905–922. doi:10.1016/S0360-5442(02)00025-7.

[63] W. Peng, Q. Wu, P. Tu, Effects of temperature and holding time on production of renewable fuels from pyrolysis of Chlorella protothecoides, J. Appl. Phycol. 12 (2000) 147–152. doi:10.1023/A:1008115025002.

[64] P. Pan, C. Hu, W. Yang, Y. Li, L. Dong, L. Zhu, D. Tong, R. Qing, Y. Fan, The direct pyrolysis and catalytic pyrolysis of Nannochloropsis sp. residue for renewable bio-oils, Bioresour. Technol. 101 (2010) 4593–4599. doi:10.1016/J.BIORTECH.2010.01.070.

[65] D.R. Vardon, B.K. Sharma, G. V. Blazina, K. Rajagopalan, T.J. Strathmann, Thermochemical conversion of raw and defatted algal biomass via hydrothermal liquefaction and slow pyrolysis, Bioresour. Technol. 109 (2012) 178–187. doi:10.1016/J.BIORTECH.2012.01.008.

[66] S. Grierson, V. Strezov, P. Shah, Properties of oil and char derived from slow pyrolysis of Tetraselmis chui, Bioresour. Technol. 102 (2011) 8232–8240. doi:https://doi.org/10.1016/j.biortech.2011.06.010.

[67] S. Grierson, V. Strezov, G. Ellem, R. Mcgregor, J. Herbertson, Thermal characterisation of microalgae under slow pyrolysis conditions, J. Anal. Appl. Pyrolysis. 85 (2009) 118–123. doi:10.1016/j.jaap.2008.10.003.

[68] X. Miao, Q. Wu, High yield bio-oil production from fast pyrolysis by metabolic controlling of Chlorella protothecoides, J. Biotechnol. 110 (2004) 85–93. doi:10.1016/j.jbiotec.2004.01.013.

[69] X. Miao, Q. Wu, C. Yang, Fast pyrolysis of microalgae to produce renewable fuels, J. Anal. Appl. Pyrolysis. 71 (2004) 855–863. doi:10.1016/j.jaap.2003.11.004.

[70] Z. Du, Y. Li, X. Wang, Y. Wan, Q. Chen, C. Wang, X. Lin, Y. Liu, P. Chen, R. Ruan, Microwave-assisted pyrolysis of microalgae for biofuel production, Bioresour. Technol. 102 (2011) 48904896. doi:10.1016/j.biortech.2011.01.055.

[71] Z. Hu, X. Ma, C. Chen, A study on experimental characteristic of microwave-assisted pyrolysis of microalgae, Bioresour. Technol. 107 (2012) 487–493. doi:10.1016/j.biortech.2011.12.095.

[72] P. Biller, A.B. Ross, Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content, Bioresour. Technol. 102 (2011) 215–225. doi:10.1016/j.biortech.2010.06.028.

[73] S. Zou, Y. Wu, M. Yang, K. Imdad, C. Li, T. Junmao, Production and characterization of bio-oil from hydrothermal liquefaction of microalgae Dunaliella tertiolecta cake, Energy. 35 (2010) 5406–5411. doi:10.1016/J.ENERGY.2010.07.013.

[74] T.M. Brown, P. Duan, P.E. Savage, Hydrothermal liquefaction and gasification of Nannochloropsis sp., Energy and Fuels. 24 (2010) 3639–3646. doi:10.1021/ef100203u.

[75] I.T.D. Cabanelas, Z. Arbib, F.A. Chinalia, C.O. Souza, J.A. Perales, P.F. Almeida, J.I. Druzian, I.A. Nascimento, From waste to energy: Microalgae production in wastewater and glycerol, Appl. Energy. 109 (2013) 283–290. doi:10.1016/J.APENERGY.2013.04.023.

[76] Z. Baicha, M.J. Salar-García, V.M. Ortiz-Martínez, F.J. Hernández-Fernández, A.P. de los Ríos, N. Labjar, E. Lotfi, M. Elmahi, A critical review on microalgae as an alternative source for bioenergy production: A promising low cost substrate for microbial fuel cells, Fuel Process. Technol. 154 (2016) 104–116. doi:10.1016/J.FUPROC.2016.08.017.

[77] H.H. Khoo, C.Y. Koh, M.S. Shaik, P.N. Sharratt, Bioenergy co-products derived from microalgae biomass via thermochemical conversion – Life cycle energy balances and CO2 emissions, Bioresour. Technol. 143 (2013) 298–307. doi:10.1016/J.BIORTECH.2013.06.004.

[78] M. Vanthoor-Koopmans, R.H. Wijffels, M.J. Barbosa, M.H.M. Eppink, Biorefinery of microalgae for food and fuel, Bioresour. Technol. 135 (2013) 142–149. doi:10.1016/j.biortech.2012.10.135.

[79] K. Wayne, J. Ying, P. Loke, N. Hui, J. Ching, T. Chuan, D. Lee, J. Chang, Bioresource Technology Microalgae biorefinery : High value products perspectives, Bioresour. Technol. 229 (2017) 53–62. doi:10.1016/j.biortech.2017.01.006.

[80] Irwandi Jaswir, Anti-inflammatory compounds of macro algae origin: A review, J. Med. Plants Res. 5 (2011) 7146–7154. doi:10.5897/jmpr11.018.

[81] D. Jha, V. Jain, B. Sharma, A. Kant, V.K. Garlapati, Microalgae-based Pharmaceuticals and Nutraceuticals: An Emerging Field with Immense Market Potential, ChemBioEng Rev. 4 (2017) 257–272. doi:10.1002/cben.201600023.

[82] H. Begum, F.M.D. Yusoff, S. Banerjee, H. Khatoon, M. Shariff, Availability and Utilization of Pigments from Microalgae, Crit. Rev. Food Sci. Nutr. 56 (2016) 2209–2222. doi:10.1080/10408398.2013.764841.

[83] H.-W. Yen, I.-C. Hu, C.-Y. Chen, S.-H. Ho, D.-J. Lee, Microalgae-based biorefinery – From biofuels to natural products, Bioresour. Technol. 135 (2013) 166–174. doi:10.1016/J.BIORTECH.2012.10.099.

[84] K. Wichuk, S. Brynjólfsson, W. Fu, Biotechnological production of value-added carotenoids from microalgae, Bioengineered. 5 (2014) 204–208. doi:10.4161/bioe.28720.

[85] E.W. Becker, Microalgae for Human and Animal Nutrition, in: Handb. Microalgal Cult., John Wiley & Sons, Ltd, 2013: pp. 461–503. doi:10.1002/9781118567166.ch25.

[86] A. Martins, N.S. Caetano, T.M. Mata, Microalgae for biodiesel production and other applications : A review, Renew. Sustain. Energy Rev. 14 (2010) 217–232. doi:10.1016/j.rser.2009.07.020.

[87] M.C. Cerón-García, M.D. Macías-Sánchez, A. Sánchez-Mirón, F. García-Camacho, E. Molina-Grima, A process for biodiesel production involving the heterotrophic fermentation of Chlorella protothecoides with glycerol as the carbon source, Appl. Energy. 103 (2013) 341–349. doi:10.1016/j.apenergy.2012.09.054.

[88] P. Feng, Z. Deng, L. Fan, Z. Hu, Lipid accumulation and growth characteristics of Chlorella zofingiensis under different nitrate and phosphate concentrations, J. Biosci. Bioeng. 114 (2012) 405–410. doi:10.1016/j.jbiosc.2012.05.007.

[89] G. Dragone, B.D. Fernandes, A.P. Abreu, A.A. Vicente, J.A. Teixeira, Nutrient limitation as a strategy for increasing starch accumulation in microalgae, Appl. Energy. 88 (2011) 3331–3335. doi:10.1016/J.APENERGY.2011.03.012.

[90] A.P. Batista, N. Bandarra, A. Raymundo, L. Gouveia, Microalgae biomass - a potential ingredient for the food industry, in: EFFoST/EHED Jt. Conf., Lisbon, Portugal, 2007.

[91] J.C. Juan, D.A. Kartika, T.Y. Wu, T.-Y.Y. Hin, Biodiesel production from jatropha oil by catalytic and non-catalytic approaches: An overview, Bioresour. Technol. 102 (2011) 452–460. doi:10.1016/J.BIORTECH.2010.09.093.

[92] Á.-D. González-Delgado, V. Kafarov, MICROALGAE BASED BIOREFINERY: ISSUES TO CONSIDER, Tecnol. y Futur. 4 (2011) 5–22. http://www.scielo.org.co/pdf/ctyf/v4n4/v4n4a01.pdf (accessed May 16, 2019).

[93] J. Benemann, Microalgae for Biofuels and Animal Feeds, Energies. 6 (2013) 5869–5886. doi:10.3390/en6115869.

[94] X. Wang, L. Lin, H. Lu, Z. Liu, N. Duan, T. Dong, H. Xiao, B. Li, P. Xu, Microalgae cultivation and culture medium recycling by a two-stage cultivation system, Front. Environ. Sci. Eng. 12 (2018) 1–14. doi:10.1007/s11783-018-1078-z.

[95] S.-Y. Hsia, S.-K. Yang, Enhancing Algal Growth by Stimulation with LED Lighting and Ultrasound, J. Nanomater. 2015 (2015) 1–11. doi:10.1155/2015/531352.

[96] A.F. Aravantinou, I.D. Manariotis, Effect of operating conditions on Chlorococcum sp. growth and lipid production, J. Environ. Chem. Eng. 4 (2016) 1217–1223. doi:10.1016/J.JECE.2016.01.028.

[97] E. Jacob-Lopes, L.G.R. Merida, M.I. Queiroz, L.Q. Zepka, Microalgae biorefineries, in: Biomass Prod. Uses, 2015: pp. 81–106. doi:http://dx.doi.org/10.5772/57353.

[98] M. Faried, M. Samer, E. Abdelsalam, R.S. Yousef, Y.A. Attia, A.S. Ali, Biodiesel production from microalgae: Processes, technologies and recent advancements, Renew. Sustain. Energy Rev. 79 (2017) 893–913. doi:10.1016/J.RSER.2017.05.199.

[99] F. Adam, M. Abert-Vian, G. Peltier, F. Chemat, "Solvent-free" ultrasound-assisted extraction of lipids from fresh microalgae cells: A green, clean and scalable process, Bioresour. Technol. 114 (2012) 457–465. doi:10.1016/J.BIORTECH.2012.02.096.

[100] P. Biller, C. Friedman, A.B. Ross, Hydrothermal microwave processing of microalgae as a pre-treatment and extraction technique for bio-fuels and bio-products, Bioresour. Technol. 136 (2013) 188–195. doi:10.1016/J.BIORTECH.2013.02.088.

[101] D. Hernández, M. Solana, B. Riaño, M.C. García-González, A. Bertucco, Biofuels from microalgae: Lipid extraction and methane production from the residual biomass in a biorefinery approach, Bioresour. Technol. 170 (2014) 370–378. doi:10.1016/j.biortech.2014.07.109.

[102] R.L. Mendes, A.D. Reis, A.F. Palavra, Supercritical CO2 extraction of γ-linolenic acid and other lipids from Arthrospira (Spirulina)maxima: Comparison with organic solvent extraction, Food Chem. 99 (2006) 57–63. doi:10.1016/j.foodchem.2005.07.019.

[103] H. Lin, Q. Wang, Q. Shen, J. Zhan, Y. Zhao, Genetic engineering of microorganisms for biodiesel production, Bioengineered. 4 (2013) 292–304.

doi:10.4161/bioe.23114.

[104] C. Muthukumaran, G. Sharmila, N. Manojkumar, A. Gnanaprakasam, V.M. Sivakumar, Optimization and Kinetic Modeling of Biodiesel Production, Ref. Modul. Mater. Sci. Mater. Eng. (2018). doi:10.1016/B978-0-12-803581-8.10578-8.

[105] C.-L. Chen, J.-S. Change, C.-C. Huang, K.-C. Ho, P.-X. Hsiao, M.-S. Wu, A Novel Biodiesel Production Method Consisting of Oil Extraction and Transesterification from Wet Microalgae, Energy Procedia. 61 (2014) 1294–1297. doi:10.1016/J.EGYPRO.2014.11.1084.

[106] G.H. Huang, F. Chen, D. Wei, X.W. Zhang, G. Chen, Biodiesel production by microalgal biotechnology, Appl. Energy. 87 (2010) 38–46. doi:10.1016/j.apenergy.2009.06.016.

[107] D. Surendhiran, A. Razack Sirajunnisa, M. Vijay, An alternative method for production of microalgal biodiesel using novel Bacillus lipase, Biotechnology. 5 (2015) 715–725. doi:10.1007/s13205-014-0271-4.

[108] S. Behzadi, M.M. Farid, Review: examining the use of different feedstock for the production of biodiesel, Asia-Pacific J. Chem. Eng. 2 (2007) 480–486. doi:10.1002/apj.85.

[109] Y. Zhang, M.A. Dubé, D.D. McLean, M. Kates, Biodiesel production from waste cooking oil: 1. Process design and technological assessment, Bioresour. Technol. 89 (2003) 1–16. doi:10.1016/S0960-8524(03)00040-3.

[110] Y. Zhang, M.A. Dubé, D.D. McLean, M. Kates, Biodiesel production from waste cooking oil: 2. Economic assessment and sensitivity analysis, Bioresour. Technol. 90 (2003) 229–240. doi:10.1016/S0960-8524(03)00150-0.

[111] J. Van Gerpen, Biodiesel processing and production, Fuel Process. Technol. 86 (2005) 1097–1107. doi:10.1016/j.fuproc.2004.11.005.

[112] M.J. Goff, N.S. Bauer, S. Lopes, W.R. Sutterlin, G.J. Suppes, Acid-catalyzed

alcoholysis of soybean oil, J. Am. Oil Chem. Soc. 81 (2004) 415–420. doi:10.1007/s11746-004-0915-6.

[113] B.D. Wahlen, R.M. Willis, L.C. Seefeldt, Biodiesel production by simultaneous extraction and conversion of total lipids from microalgae, cyanobacteria, and wild mixed-cultures, Bioresour. Technol. 102 (2011) 2724–2730. doi:10.1016/J.BIORTECH.2010.11.026.

[114] D.-T. Tran, K.-L. Yeh, C.-L. Chen, J.-S. Chang, Enzymatic transesterification of microalgal oil from Chlorella vulgaris ESP-31 for biodiesel synthesis using immobilized Burkholderia lipase, Bioresour. Technol. 108 (2012) 119–127. doi:10.1016/J.BIORTECH.2011.12.145.

[115] K.M. Eyster, The membrane and lipids as integral participants in signal transduction: lipid signal transduction for the non-lipid biochemist, Adv. Physiol. Educ. 31 (2007) 5–16. doi:10.1152/advan.00088.2006.

[116] S.L. Holdt, S. Kraan, Bioactive compounds in seaweed: functional food applications and legislation, J. Appl. Phycol. 23 (2011) 543–597. doi:10.1007/s10811-010-9632-5.

[117] J.L. García, M. de Vicente, B. Galán, Microalgae, old sustainable food and fashion nutraceuticals, Microb. Biotechnol. 10 (2017) 1017–1024. doi:10.1111/1751-7915.12800.

[118] M.P. Caporgno, A. Mathys, Trends in Microalgae Incorporation Into Innovative Food Products With Potential Health Benefits, Front. Nutr. 5 (2018) 1–10. doi:10.3389/fnut.2018.00058.

[119] B. da S. Vaz, J.B. Moreira, M.G. de Morais, J.A.V. Costa, Microalgae as a new source of bioactive compounds in food supplements, Curr. Opin. Food Sci. 7 (2016) 73–77. doi:10.1016/j.cofs.2015.12.006.

[120] G.Y. Sun, A. Simonyi, K.L. Fritsche, D.Y. Chuang, M. Hannink, Z. Gu, C.M. Greenlief,

J.K. Yao, J.C. Lee, D.Q. Beversdorf, Docosahexaenoic acid (DHA): An essential nutrient and a nutraceutical for brain health and diseases, Prostaglandins Leukot. Essent. Fat. Acids. 136 (2018) 3–13. doi:10.1016/j.plefa.2017.03.006.

[121] F. Echeverría, R. Valenzuela, M. Catalina Hernandez-Rodas, A. Valenzuela, Docosahexaenoic acid (DHA), a fundamental fatty acid for the brain: New dietary sources, Prostaglandins Leukot. Essent. Fat. Acids. 124 (2017) 1–10. doi:10.1016/j.plefa.2017.08.001.

[122] T.C. Adarme-Vega, D.K.Y. Lim, M. Timmins, F. Vernen, Y. Li, P.M. Schenk, Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production, Microb. Cell Fact. 11 (2012) 1–10. doi:10.1186/1475-2859-11-96.

[123] D. Rossoll, R. Bermúdez, H. Hauss, K.G. Schulz, U. Riebesell, U. Sommer, M. Winder, Ocean Acidification-Induced Food Quality Deterioration Constrains Trophic Transfer, PLoS One. 7 (2012) 1–6. doi:10.1371/journal.pone.0034737.

[124] E. Ryckebosch, C. Bruneel, R. Termote-Verhalle, K. Goiris, K. Muylaert, I. Foubert, Nutritional evaluation of microalgae oils rich in omega-3 long chain polyunsaturated fatty acids as an alternative for fish oil, Food Chem. 160 (2014) 393–400. doi:https://doi.org/10.1016/j.foodchem.2014.03.087.

[125] V. Chochois, D. Dauvillée, A. Beyly, D. Tolleter, S. Cuiné, H. Timpano, S. Ball, L. Cournac, G. Peltier, Hydrogen Production in Chlamydomonas: Photosystem II-Dependent and -Independent Pathways Differ in Their Requirement for Starch Metabolism, Plant Physiol. 151 (2009) 631–640. doi:10.1104/pp.109.144576.

[126] I.A. Schepetkin, M.T. Quinn, Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential, Int. Immunopharmacol. 6 (2006) 317–333. doi:10.1016/J.INTIMP.2005.10.005.

[127] J.K. Park, Z.-H. Kim, C.G. Lee, A. Synytsya, H.S. Jo, S.O. Kim, J.W. Park, Y. Il Park, Characterization and immunostimulating activity of a water-soluble polysaccharide

isolated from Haematococcus lacustris, Biotechnol. Bioprocess Eng. 16 (2011) 1090–1098. doi:10.1007/s12257-011-0173-9.

[128] T. Tannin-Spitz, M. Bergman, D. van-Moppes, S. Grossman, S. (Malis) Arad, Antioxidant activity of the polysaccharide of the red microalga Porphyridium sp, J. Appl. Phycol. 17 (2005) 215–222. doi:10.1007/s10811-005-0679-7.

[129] M.S. Matsui, N. Muizzuddin, S. Arad, K. Marenus, Sulfated polysaccharides from red microalgae have antiinflammatory properties in vitro and in vivo, Appl. Biochem. Biotechnol. 104 (2003) 13–22. doi:10.1385/ABAB:104:1:13.

[130] M. Kim, J.H. Yim, S.-Y. Kim, H.S. Kim, W.G. Lee, S.J. Kim, P.-S. Kang, C.-K. Lee, In vitro inhibition of influenza A virus infection by marine microalga-derived sulfated polysaccharide p-KG03, Antiviral Res. 93 (2012) 253–259. doi:https://doi.org/10.1016/j.antiviral.2011.12.006.

[131] S. Guzmán, A. Gato, M. Lamela, M. Freire-Garabal, J.M. Calleja, Anti-inflammatory and immunomodulatory activities of polysaccharide from Chlorella stigmatophora and Phaeodactylum tricornutum, Phyther. Res. 17 (2003) 665–670. doi:10.1002/ptr.1227.

[132] Z.A. Mohamed, Polysaccharides as a protective response against microcystin-induced oxidative stress in Chlorella vulgaris and Scenedesmus quadricauda and their possible significance in the aquatic ecosystem, Ecotoxicology. 17 (2008) 504. doi:10.1007/s10646-008-0204-2.

[133] M. Kim, J.H. Yim, S.-Y. Kim, H.S. Kim, W.G. Lee, S.J. Kim, P.-S. Kang, C.-K. Lee, In vitro inhibition of influenza A virus infection by marine microalga-derived sulfated polysaccharide p-KG03, Antiviral Res. 93 (2012) 253–259. doi:10.1016/J.ANTIVIRAL.2011.12.006.

[134] C.-K. Lee, H.S. Kim, J.R. Nam, M.-J. Lee, J.-H. Yim, H.K. Lee, E. De Clercq, Anti-Picornavirus Activity and Other Antiviral Activity of Sulfated Exopolysaccharide

from the Marine Microalga Gyrodinium impudicum Strain KG03, Antiviral Res. 82 (2009) A40. doi:10.1016/J.ANTIVIRAL.2009.02.084.

[135] M.S. Matsui, N. Muizzuddin, S. Arad, K. Marenus, Sulfated Polysaccharides from Red Microalgae Have Antiinflammatory Properties In Vitro and In Vivo, Appl. Biochem. Biotechnol. 104 (2003) 13–22. https://link.springer.com/content/pdf/10.1385/ABAB:104:1:13.pdf (accessed May 23, 2019).

[136] B. Chen, W. You, J. Huang, Y. Yu, W. Chen, Isolation and antioxidant property of the extracellular polysaccharide from Rhodella reticulata, World J. Microbiol. Biotechnol. 26 (2010) 833–840. doi:10.1007/s11274-009-0240-y.

[137] J.R. Miranda, P.C. Passarinho, L. Gouveia, Bioethanol production from Scenedesmus obliquus sugars: the influence of photobioreactors and culture conditions on biomass production, Appl. Microbiol. Biotechnol. 96 (2012) 555–564. doi:10.1007/s00253-012-4338-z.

[138] C.-Y. Chen, X.-Q. Zhao, H.-W. Yen, S.-H. Ho, C.-L. Cheng, F.-W. Bai, Microalgae-based carbohydrates for biofuel production, Biochem. Eng. J. 78 (2013) 1–10. doi:10.1016/J.BEJ.2013.03.006.

[139] C. Simas-Rodrigues, H.D.M. Villela, A.P. Martins, L.G. Marques, P. Colepicolo, A.P. Tonon, Microalgae for economic applications: advantages and perspectives for bioethanol, J. Exp. Bot. 66 (2015) 4097–4108. doi:10.1093/jxb/erv130.

[140] Y. Ueno, N. Kurano, S. Miyachi, Ethanol production by dark fermentation in the marine green alga, Chlorococcum littorale, J. Ferment. Bioeng. 86 (1998) 38–43. doi:10.1016/S0922-338X(98)80031-7.

[141] G. Markou, I. Angelidaki, D. Georgakakis, Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels, Appl. Microbiol. Biotechnol. 96 (2012) 631–

645. doi:10.1007/s00253-012-4398-0.

[142] A. Hirano, R. Ueda, S. Hirayama, Y. Ogushi, CO2 fixation and ethanol production with microalgal photosynthesis and intracellular anaerobic fermentation, Energy. 22 (1997) 137–142. doi:10.1016/S0360-5442(96)00123-5.

[143] S. (Malis) Arad, O. Levy-Ontman, Red microalgal cell-wall polysaccharides: biotechnological aspects, Curr. Opin. Biotechnol. 21 (2010) 358–364. doi:10.1016/J.COPBIO.2010.02.008.

[144] M. De Vries, I.J.M. De Boer, Comparing environmental impacts for livestock products: A review of life cycle assessments, Livest. Sci. 128 (2009) 1–11. doi:10.1016/j.livsci.2009.11.007.

[145] S. Smetana, M. Sandmann, S. Rohn, D. Pleissner, V. Heinz, Autotrophic and heterotrophic microalgae and cyanobacteria cultivation for food and feed: life cycle assessment, Bioresour. Technol. 245 (2017) 162–170. doi:10.1016/j.biortech.2017.08.113.

[146] A.K. Koyande, K.W. Chew, J. Lim, S.Y. Lee, M.K. Lam, P. Show, Optimization of protein extraction from Chlorella Vulgaris via novel sugaring-out assisted liquid biphasic electric flotation system, Eng. Life Sci. 19 (2019) 968–977. doi:10.1002/elsc.201900068.

[147] W.J. Bjornsson, K.M. MacDougall, J.E. Melanson, S.J.B. O'Leary, P.J. McGinn, Pilot-scale supercritical carbon dioxide extractions for the recovery of triacylglycerols from microalgae: a practical tool for algal biofuels research, J. Appl. Phycol. 24 (2012) 547–555. doi:10.1007/s10811-011-9756-2.

[148] H. Begum, F.M. Yusoff, S. Banerjee, H. Khatoon, M. Shariff, Availability and Utilization of Pigments from Microalgae Availability and Utilization of Pigments from Microalgae, Crit. Rev. Food Sci. Nutr. 56 (2015) 2209–2222. doi:10.1080/10408398.2013.764841.

[149] S. Kobylewski, M.F. Jacobson, Food Dyes A Rainbow of Risks, University of California, Los Angeles, 2010. https://cspinet.org/sites/default/files/attachment/food-dyes-rainbow-of-risks.pdf (accessed October 12, 2018).

[150] R.S. Parmar, C. Singh, A comprehensive study of eco-friendly natural pigment and its applications, Biochem. Biophys. Reports. 13 (2018) 22–26. doi:10.1016/j.bbrep.2017.11.002.

[151] G. Farré, G. Sanahuja, S. Naqvi, C. Bai, T. Capell, C. Zhu, P. Christou, Travel advice on the road to carotenoids in plants, Plant Sci. 179 (2010) 28–48. doi:10.1016/j.plantsci.2010.03.009.

[152] K.J.M. Mulders, Phototrophic pigment production with microalgae, Wageningen University, 2014. http://edepot.wur.nl/323615 (accessed October 12, 2018).

[153] B. Wang, Y. Li, N. Wu, C.Q. Lan, CO2 bio-mitigation using microalgae, Appl. Microbiol. Biotechnol. 79 (2008) 707–718. doi:10.1007/s00253-008-1518-y.

[154] N. Hanagata, T. Takeuchi, Y. Fukukj, D.J. Barnes, I. Karube, TOLERANCE OF MICROALGAE TO HIGH CO, AND HIGH TEMPERATURE, Phytochemistry. 31 (1992) 3345–3348. https://ac.els-cdn.com/003194229283682O/1-s2.0-003194229283682O-main.pdf?_tid=8ee73d4a-c623-4327-bf18-6f52c4877a73&acdnat=1543813543_d35f685f64ecb1821b8b94e6c2e02d69 (accessed December 3, 2018).

[155] Z. Wu, S. Wu, X. Shi, SUPERCRITICAL FLUID EXTRACTION AND DETERMINATION OF LUTEIN IN HETEROTROPHICALLY CULTIVATED CHLORELLA PYRENOIDOSA, 2007. https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1745-4530.2007.00102.x (accessed November 30, 2018).

[156] S. Takaichi, Carotenoids in Algae: Distributions, Biosyntheses and Functions, Mar. Drugs. 9 (2011) 1101–1118. doi:10.3390/md9061101.

[157] R. Sathasivam, R. Radhakrishnan, A. Hashem, E.F. Abd_Allah, Microalgae metabolites: A rich source for food and medicine, Saudi J. Biol. Sci. (2017). doi:10.1016/j.sjbs.2017.11.003.

[158] R. Sathasivam, N. Juntawong, Modified medium for enhanced growth of Dunaliella strains, INT J CURR SCI. 5 (2013) 67–73. http://www.currentsciencejournal.info/issuespdf/Ramaraj.pdf (accessed November 27, 2018).

[159] D.B. Rodrigues, C.R. Menezes, A.Z. Mercadante, E. Jacob-Lopes, L.Q. Zepka, Bioactive pigments from microalgae Phormidium autumnale, Food Res. Int. 77 (2015) 273–279. doi:10.1016/j.foodres.2015.04.027.

[160] W.J. Henley, K.M. Major, J.L. Hironaka, RESPONSE TO SALINITY AND HEAT STRESS IN TWO HALOTOLERANT CHLOROPHYTE ALGAE, J. Phycol. 38 (n.d.) 757–766. doi:10.1046/j.1529-8817.2002.01172.x.

[161] R. Raja, C. Anbazhagan, D. Lakshmi, R. Rengasamy, Nutritional studies on Dunaliella salina (Volvocales, Chlorophyta) under laboratory conditions, Seaweed Res Util. 26 (2004) 127–146.

[162] M.A. Borowitzka, High-value products from microalgae---their development and commercialisation, J. Appl. Phycol. 25 (2013) 743–756. doi:10.1007/s10811-013-9983-9.

[163] M.A. Gammone, G. Riccioni, N.D.' Orazio, Carotenoids: potential allies of cardiovascular health?, Food Nutr. Res. (2015). doi:10.3402/fnr.v59.26762.

[164] G. Riccioni, N. D'orazio, N. Palumbo, V. Bucciarelli, E. Di Ilio, L.A. Bazzano, T. Bucciarelli, Relationship between plasma antioxidant concentrations and carotid intima-media thickness: the Asymptomatic Carotid Atherosclerotic Disease In Manfredonia Study, Eur. J. Cardiovasc. Prev. Rehabil. 16 (2009) 351–357. doi:10.1097/HJR.0b013e328325d807.

[165] R.T. Lorenz, G.R. Cysewski, Commercial potential for Haematococcus microalgae as a natural source of astaxanthin, Trends Biotechnol. 18 (2000) 160–167. doi:10.1016/S0167-7799(00)01433-5.

[166] D. Pelah, A. Sintov, E. Cohen, The Effect of Salt Stress on the Production of Canthaxanthin and Astaxanthin by Chlorella zofingiensis Grown Under Limited Light Intensity, World J. Microbiol. Biotechnol. 20 (2004) 483–486. doi:10.1023/B:WIBI.0000040398.93103.21.

[167] P.-F. Ip, F. Chen, Employment of reactive oxygen species to enhance astaxanthin formation in Chlorella zofingiensis in heterotrophic culture, Process Biochem. 40 (2005) 3491–3496. doi:10.1016/j.procbio.2005.02.014.

[168] I. Higuera-Ciapara, L. Félix-Valenzuela, F.M. Goycoolea, L. F ´ Elix-Valenzuela, Astaxanthin: A Review of its Chemistry and Applications, Crit. Rev. Food Sci. Nutr. 46 (2006) 185–196. doi:10.1080/10408690590957188.

[169] M. Guerin, M.E. Huntley, M. Olaizola, Haematococcus astaxanthin: applications for human health and nutrition, TRENDS Biotechnol. 21 (2003) 210–216. doi:10.1016/S0167-7799(03)00078-7.

[170] J.S. Park, J.H. Chyun, Y.K. Kim, L.L. Line, B.P. Chew, Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans, Nutr. Metab. (Lond). 7 (2010) 18. doi:10.1186/1743-7075-7-18.

[171] J.H. Kim, M.J. Chang, H.D. Choi, Y.-K. Youn, J.T. Kim, J.M. Oh, W.G. Shin, Protective Effects of Haematococcus Astaxanthin on Oxidative Stress in Healthy Smokers, J. Med. Food. 14 (2011) 1469–1475. doi:10.1089/jmf.2011.1626.

[172] H. Yoshida, H. Yanai, K. Ito, Y. Tomono, T. Koikeda, H. Tsukahara, N. Tada, Administration of natural astaxanthin increases serum HDL-cholesterol and adiponectin in subjects with mild hyperlipidemia, Atherosclerosis. 209 (2010) 520–523. doi:10.1016/j.atherosclerosis.2009.10.012.

[173] K. Goiris, K. Muylaert, I. Fraeye, I. Foubert, J. De Brabanter, L. De Cooman, Antioxidant potential of microalgae in relation to their phenolic and carotenoid content, J. Appl. Phycol. 24 (2012) 1477–1486. doi:10.1007/s10811-012-9804-6.

[174] A. Maadane, N. Merghoub, T. Ainane, H. El Arroussi, R. Benhima, S. Amzazi, Y. Bakri, I. Wahby, Antioxidant activity of some Moroccan marine microalgae: Pufa profiles, carotenoids and phenolic content, J. Biotechnol. 215 (2015) 13–19. doi:https://doi.org/10.1016/j.jbiotec.2015.06.400.

[175] W.M. Bishop, H.M. Zubeck, Evaluation of Microalgae for use as Nutraceuticals and Nutritional Supplements, J. Nutr. Food Sci. 2 (2012). doi:10.4172/2155-9600.1000147.

[176] N. Seyidoglu, S. Inan, C. Aydin, A Prominent Superfood: Spirulina platensis, Intech Open. 2 (2014) 64. doi:10.5772/32009.

[177] S. Nagini, F. Palitti, A.T. Natarajan, Chemopreventive Potential of Chlorophyllin: A Review of the Mechanisms of Action and Molecular Targets, Nutr. Cancer. 67 (2015) 203–211. doi:10.1080/01635581.2015.990573.

[178] J. Das, A. Samadder, J. Mondal, S.K. Abraham, A. Rahman Khuda-Bukhsh, Nano-encapsulated chlorophyllin significantly delays progression of lung cancer both in in vitro and in vivo models through activation of mitochondrial signaling cascades and drug-DNA interaction, Environ. Toxicol. Pharmacol. 46 (2016) 147–157. doi:10.1016/j.etap.2016.07.006.

[179] R. Raghav Sonani, R. Prasad Rastogi, R. Patel, D. Madamwar Ravi Raghav Sonani, D. Madamwar, Recent advances in production, purification and applications of phycobiliproteins, World J Biol Chem. 7 (2016) 100–109. doi:10.4331/wjbc.v7.i1.100.

[180] S. Sekar, M. Chandramohan, Phycobiliproteins as a commodity: trends in applied research, patents and commercialization, J. Appl. Phycol. 20 (2008) 113–136.

doi:10.1007/s10811-007-9188-1.

[181] F. Depeint, W. Robert Bruce, N. Shangari, R. Mehta, P.J. O'brien, Mitochondrial function and toxicity: Role of the B vitamin family on mitochondrial energy metabolism, Chem. Biol. Interact. 163 (2006) 94–112. doi:10.1016/j.cbi.2006.04.014.

[182] J. Fabregas, C. Herrero, Vitamin content of four marine microalgae. Potential use as source of vitamins in nutrition, J. Ind. Microbiol. 5 (1990) 259–263. doi:10.1007/BF01569683.

[183] M.N. Islam, F. Alsenani, P.M. Schenk, Microbial Functional Foods and Nutraceuticals, John Wiley & Sons Ltd., 2017. doi:https://doi.org/10.1002/9781119048961.ch1.

[184] F. Watanabe, S. Takenaka, H. Kittaka-Katsura, S. Ebara, E. Miyamoto, Characterization and Bioavailability of Vitamin B12-Compounds from Edible Algae, J Nutr Sci Vitaminoi. 48 (2002) 325–331. doi:10.3177/jnsv.48.325.

[185] M.T. Croft, A.D. Lawrence, E. Raux-Deery, M.J. Warren, A.G. Smith, Algae acquire vitamin B12through a symbiotic relationship with bacteria, Nature. 438 (2005) 90–93. doi:10.1038/nature04056.

[186] L.H. Allen, Causes of vitamin B 12 and folate deficiency, 29 (2008) 20–34.

[187] J. Ortiz, E. Uquiche, P. Robert, N. Romero, V. Quitral, C. Llantén, Functional and nutritional value of the Chilean seaweeds Codium fragile, Gracilaria chilensis and Macrocystis pyrifera, Eur. J. Lipid Sci. Technol. 111 (2009) 320–327. doi:10.1002/ejlt.200800140.

[188] P. Ferraces-Casais, M.A. Lage-Yusty, A. Rodríguez-Bernaldo De Quirós, J. López-Hernández, Evaluation of Bioactive Compounds in Fresh Edible Seaweeds, Food Anal. Methods. 5 (2012) 828–834. doi:10.1007/s12161-011-9321-2.

[189] G. Hernández-Carmona, S. Carrillo-Domínguez, D.L. Arvizu-Higuera, Y.E. Rodríguez-Montesinos, J.I. Murillo-Álvarez, M. Muñoz-Ochoa, R.M. Castillo-

Domínguez, Monthly variation in the chemical composition of Eisenia arborea J.E. Areschoug, J. Appl. Phycol. 21 (2009) 607–616. doi:10.1007/s10811-009-9454-5.

[190] S. Škrovánková, Seaweed Vitamins as Nutraceuticals, Adv. Food Nutr. Res. 64 (2011) 357–369. doi:10.1016/B978-0-12-387669-0.00028-4.

[191] S. Takenaka, S. Sugiyama, S. Ebara, E. Miyamoto, K. Abe, Y. Tamura, F. Watanabe, S. Tsuyama, Y. Nakano, Feeding dried purple laver (nori) to vitamin B 12-deficient rats significantly improves vitamin B 12 status, Br. J. Nutr. 85 (2001) 699–703. doi:10.1079/BJN2001352.

[192] V. Hajhashemi, G. Vaseghi, M. Pourfarzam, A. Abdollahi, Are antioxidants helpful for disease prevention?, Res. Pharm. Sci. 5 (2010) 1–8. http://www.ncbi.nlm.nih.gov/pubmed/21589762 (accessed November 8, 2018).

[193] L.A. Pham-Huy, H. He, C. Pham-Huy, Free Radicals, Antioxidants in Disease and Health, Int. J. Biomed. Sci. 4 (2008) 89–96.

[194] S. Topdag, A. Aslaner, C. Tataroglu, Z. Ilce, Evaluation of antioxidant capacity in lung carcinoma, Indian J. Thorac. Cardiovasc. Surg. 21 (2005) 269–271. doi:10.1007/s12055-005-0004-8.

[195] K.K. Kattappagari, R.T. CS, R.K. Kommalapati, C. Poosarla, S.R. Gontu, B.V.R. Reddy, Role of antioxidants in facilitating the body functions: A review, J. OfOrofacial Sci. 7 (2015) 71–75. doi:10.4103/0975-8844.169745.

[196] A.P. Batista, L. Gouveia, N.M. Bandarra, J.M. Franco, A. Raymundo, Comparison of microalgal biomass profiles as novel functional ingredient for food products, Algal Res. 2 (2013) 164–173. doi:10.1016/j.algal.2013.01.004.

[197] R. Matsukawa, M. Hotta, Y. Masuda, M. Chihara, I. Karube, Antioxidants from carbon dioxide fixing Chlorella sorokiniana, 2000. https://link.springer.com/content/pdf/10.1023%2FA%3A1008141414115.pdf (accessed November 28, 2018).

[198] F.M.I. Natrah, F.M. Yusoff, M. Shariff, F. Abas, N.S. Mariana, Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value, J. Appl. Phycol. 19 (2007) 711–718. doi:10.1007/s10811-007-9192-5.

[199] R.H. Wijffels, M.J. Barbosa, An Outlook on Microalgal Biofuels, Science (80-.). 329 (2010) 796–799. doi:10.1126/science.1189003.

[200] Algae Products Market By Application (Nutraceuticals, Food & Feed Supplements, Pharmaceuticals, Paints & Colorants, Pollution Control, Others) - Growth, Future Prospects, Competitive Analysis, and Forecast 2016 - 2023, 2017. https://www.credenceresearch.com/report/algae-products-market (accessed November 30, 2018).

[201] P. Singh, R. Singh, A. Jha, P. Rasane, A.K. Gautam, Optimization of a process for high fibre and high protein biscuit, J. Food Sci. Technol. 52 (2015) 1394–1403. doi:10.1007/s13197-013-1139-z.

[202] H.H. Abd El Baky, G.S. El Baroty, E.A. Ibrahem, H.H. Abd, E. Baky, Functional characters evaluation of biscuits sublimated with pure phycocyanin isolated from Spirulina and Spirulina biomass, Nutr. Hosp. 32 (2015) 231–241. doi:10.3305/nh.2015.32.1.8804.

[203] A.P. Batista, A. Niccolai, P. Fradinho, S. Fragoso, I. Bursic, L. Rodolfi, N. Biondi, M.R. Tredici, I. Sousa, A. Raymundo, Microalgae biomass as an alternative ingredient in cookies: Sensory, physical and chemical properties, antioxidant activity and in vitro digestibility, Algal Res. 26 (2017) 161–171. doi:10.1016/j.algal.2017.07.017.

[204] L. Gouveia, C. Coutinho, E. Mendonça, A.P. Batista, I. Sousa, N.M. Bandarra, A. Raymundo, Functional biscuits with PUFA-ω3 from Isochrysis galbana, J. Sci. Food Agric. 88 (2008) 891–896. doi:10.1002/jsfa.3166.

[205] Y.A. Hafsa, D. Amel, S. Samia, S. Sidahmed, Evaluation of nutritional and sensory

properties of bread enriches with Spirulina, 2014. www.afst.valahia.ro (accessed January 7, 2019).

[206] B. Ak, E. Avşaroğlu, O. Işık, G. Özyurt, E. Kafkas, M. Etyemez, L. Uslu, Nutritional and physicochemical characteristics of bread enriched with microalgae Spirulina platensis, Int. J. Eng. Res. Appl. 6 (2016) 30–38. www.ijera.com (accessed January 7, 2019).

[207] F. da Silva Figuerira, C. Rubira Silva, Elaboration of gluten-free bread enriched with microalgae Spirulina plantesis, Brazilian J. Food Technol. 14 (2011) 308–316. doi:10.4260/BJFT2011140400037.

[208] M. Dinu, G. Vlasceanu, A. Dune, G. Rotaru, Researches concerning the growth of nutritive value of the bread products through the Spirulina adding, J. Environ. Prot. Ecol. 13 (2012) 660–665. http://www.jepe-journal.info/vol-13-no-2 (accessed January 7, 2019).

[209] P. García-Segovia, M.J. Pagán-Moreno, I.F. Lara, J. Martínez-Monzó, Effect of microalgae incorporation on physicochemical and textural properties in wheat bread formulation, Food Sci. Technol. Int. 23 (2017) 437–447. doi:10.1177/1082013217700259.

[210] L. Gouveia, A.P. Batista, A. Miranda, J. Empis, A. Raymundo, Chlorella vulgaris biomass used as colouring source in traditional butter cookies, Innov. Food Sci. Emerg. Technol. 8 (2007) 433–436. doi:10.1016/j.ifset.2007.03.026.

[211] A.K.M.M. Hossain, M.A. Brennan, S.L. Mason, X. Guo, X.A. Zeng, C.S. Brennan, The Effect of Astaxanthin-Rich Microalgae "Haematococcus pluvialis" and Wholemeal Flours Incorporation in Improving the Physical and Functional Properties of Cookies, Foods. 6 (2017). doi:10.3390/foods6080057.

[212] B.F. Lucas, M.G. de Morais, T.D. Santos, J.A.V. Costa, Spirulina for snack enrichment: Nutritional, physical and sensory evaluations, LWT- Food Sci. Technol.

90 (2018) 270–276. doi:10.1016/j.lwt.2017.12.032.

[213] A. Raymundo, L. Gouveia, A.P. Batista, J. Empis, I. Sousa, Fat mimetic capacity of Chlorella vulgaris biomass in oil-in-water food emulsions stabilized by pea protein, Food Res. Int. 38 (2005) 961–965. doi:10.1016/j.foodres.2005.02.016.

[214] L. Gouveia, A. Raymundo, A.P. Batista, I. Sousa, J. Empis, Chlorella vulgaris and Haematococcus pluvialis biomass as colouring and antioxidant in food emulsions, Eur. Food Res. Technol. 222 (2005) 362. doi:10.1007/s00217-005-0105-z.

[215] L. Varga, J. Szigeti, R. Ková, T. Fö, S. Buti, Influence of a Spirulina platensis Biomass on the Microflora of Fermented ABT Milks During Storage (R1), J. Dairy Sci. 85 (2002) 1031–1038. doi:10.3168/jds.S0022-0302(02)74163-5.

[216] H. Beheshtipour, A.M. Mortazavian, R. Mohammadi, S. Sohrabvandi, K. Khosravi-Darani, Supplementation of Spirulina platensis and Chlorella vulgaris Algae into Probiotic Fermented Milks, Compr. Rev. Food Sci. Food Saf. 12 (2013) 144–154. doi:10.1111/1541-4337.12004.

[217] M. Fradique, A.P. Batista, M.C. Nunes, L. Gouveia, N.M. Bandarra, A. Raymundo, Incorporation of Chlorella vulgaris and Spirulina maxima biomass in pasta products. Part 1: Preparation and evaluation, J. Sci. Food Agric. 90 (2010) 1656–1664. doi:10.1002/jsfa.3999.

[218] N. Zouari, M. Abid, N. Fakhfakh, M.A. Ayadi, L. Zorgui, M. Ayadi, H. Attia, Blue-green algae (Arthrospira platensis) as an ingredient in pasta: free radical scavenging activity, sensory and cooking characteristics evaluation, Int. J. Food Sci. Nutr. 62 (2011) 811–813. doi:10.3109/09637486.2011.582461.

[219] E.R. De Marco, M.E. Steffolani, C.S. Martínez, A.E. León, Effects of spirulina biomass on the technological and nutritional quality of bread wheat pasta, LWT - Food Sci. Technol. 58 (2014) 102–108. doi:https://doi.org/10.1016/j.lwt.2014.02.054.

[220] F.K. El-Baz, S.M. Abdo, A.M.S. Hussein, Microalgae Dunaliella salina for use as Food Supplement to improve Pasta Quality, Int. J. Pharm Sci Rev. 46 (2017) 45–51. http://globalresearchonline.net/journalcontents/v46-2/10.pdf (accessed January 7, 2019).

[221] M. Fradique, A.P. Batista, M.C. Nunes, L. Gouveia, N.M. Bandarra, A. Raymundo, Isochrysis galbana and Diacronema vlkianum biomass incorporation in pasta products as PUFA's source, LWT - Food Sci. Technol. 50 (2013) 312–319. doi:https://doi.org/10.1016/j.lwt.2012.05.006.

[222] A.C. Lemes, K. Pereira Takeuchi, J. Carlos, M. De Carvalho, E. Dalva, G. Danesi, Fresh Pasta Production Enriched with Spirulina platensis Biomass, Brazilian Arch. Biol. Technol. 55 (2012) 741–750. http://www.scielo.br/pdf/babt/v55n5/14.pdf (accessed January 7, 2019).

[223] J. Jeon, Effect of Chlorella Addition on the Quality of Processed Cheese, J. Korean Soc. Food Sci. Nutr. 35 (2006) 373–377. http://www.dbpia.co.kr/Article/NODE00704631.

[224] L. Gouveia, A.P. Batista, A. Raymundo, N. Bandarra, Spirulina maxima and Diacronema vlkianum microalgae in vegetable gelled desserts, Nutr. Food Sci. 38 (2008) 492–501. doi:10.1108/00346650810907010.

[225] A.P. Batista, L. Gouveia, M.C. Nunes, J.M. Franco, A. Raymundo, Microalgae Biomass as a Novel Functional Ingredient in Mixed Gel Systems, in: Gums Stab. Food Ind. 14, The Royal Society of Chemistry, 2008: pp. 487–494. doi:10.1039/9781847558312-00487.

[226] A.P. Batista, M.C. Nunes, A. Raymundo, L. Gouveia, I. Sousa, F. Cordobés, A. Guerrero, J.M. Franco, Microalgae biomass interaction in biopolymer gelled systems, Food Hydrocoll. 25 (2011) 817–825. doi:10.1016/j.foodhyd.2010.09.018.

[227] E.-J. Cho, E.-S. Nam, S.-I. Park, Keeping quality and sensory properties

ofdrinkable yoghurt with added Chlorella extract. Korean J Food Nutr. (2004) 17:128–32. Available online, Korean J. Food Nutr. 17 (2004) 128–137. http://www.koreascience.or.kr/article/JAKO200411922361896.page.

[228] L. Barsanti, P. Gualtieri, Is exploitation of microalgae economically and energetically sustainable?, Algal Res. 31 (2018) 107–115. doi:10.1016/J.ALGAL.2018.02.001.

[229] O. Jorquera, A. Kiperstok, E.A. Sales, M. Embiruçu, M.L. Ghirardi, Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors, Bioresour. Technol. 101 (2010) 1406–1413. doi:10.1016/J.BIORTECH.2009.09.038.

[230] M.R. Tredici, N. Bassi, M. Prussi, N. Biondi, L. Rodolfi, G. Chini Zittelli, G. Sampietro, Energy balance of algal biomass production in a 1-ha "Green Wall Panel" plant: How to produce algal biomass in a closed reactor achieving a high Net Energy Ratio, Appl. Energy. 154 (2015) 1103–1111. doi:10.1016/J.APENERGY.2015.01.086.

[231] E.P. Bennion, D.M. Ginosar, J. Moses, F. Agblevor, J.C. Quinn, Lifecycle assessment of microalgae to biofuel: Comparison of thermochemical processing pathways, Appl. Energy. 154 (2015) 1062–1071. doi:10.1016/J.APENERGY.2014.12.009.

[232] R. Chowdhury, M. Franchetti, Life cycle energy demand from algal biofuel generated from nutrients present in the dairy waste, Sustain. Prod. Consum. 9 (2017) 22–27. doi:10.1016/J.SPC.2016.07.001.

[233] L. Soh, M. Montazeri, B.Z. Haznedaroglu, C. Kelly, J. Peccia, M.J. Eckelman, J.B. Zimmerman, Evaluating microalgal integrated biorefinery schemes: Empirical controlled growth studies and life cycle assessment, Bioresour. Technol. 151 (2014) 19–27. doi:10.1016/J.BIORTECH.2013.10.012.

[234] J. Hoffman, R.C. Pate, T. Drennen, J.C. Quinn, Techno-economic assessment of open microalgae production systems, Algal Res. 23 (2017) 51–57.

doi:10.1016/j.algal.2017.01.005.

[235] Y.K. Dasan, M.K. Lam, S. Yusup, J.W. Lim, K.T. Lee, Life cycle evaluation of microalgae biofuels production: Effect of cultivation system on energy, carbon emission and cost balance analysis, Sci. Total Environ. 688 (2019) 112–128. doi:10.1016/j.scitotenv.2019.06.181.

[236] G.P. 't Lam, M.H. Vermuë, M.H.M. Eppink, R.H. Wijffels, C. van den Berg, Multi-Product Microalgae Biorefineries: From Concept Towards Reality, Trends Biotechnol. 36 (2018) 216–227. doi:10.1016/j.tibtech.2017.10.011.

[237] I. Gifuni, A. Pollio, C. Safi, A. Marzocchella, G. Olivieri, Current Bottlenecks and Challenges of the Microalgal Biorefinery, Trends Biotechnol. 37 (2019) 242–252. doi:10.1016/J.TIBTECH.2018.09.006.

[238] A.L. Lupatini, L. de Oliveira Bispo, L.M. Colla, J.A.V. Costa, C. Canan, E. Colla, Protein and carbohydrate extraction from S. platensis biomass by ultrasound and mechanical agitation, Food Res. Int. 99 (2017) 1028–1035. doi:10.1016/J.FOODRES.2016.11.036.

[239] B. Gilbert-López, J.A. Mendiola, L.A.M. van den Broek, B. Houweling-Tan, L. Sijtsma, A. Cifuentes, M. Herrero, E. Ibáñez, Green compressed fluid technologies for downstream processing of Scenedesmus obliquus in a biorefinery approach, Algal Res. 24 (2017) 111–121. doi:10.1016/J.ALGAL.2017.03.011.

[240] T. Dong, E.P. Knoshaug, R. Davis, L.M.L. Laurens, S. Van Wychen, P.T. Pienkos, N. Nagle, Combined algal processing: A novel integrated biorefinery process to produce algal biofuels and bioproducts, Algal Res. 19 (2016) 316–323. doi:10.1016/J.ALGAL.2015.12.021.

[241] N. Misra, P.K. Panda, B.K. Parida, B.K. Mishra, Way forward to achieve sustainable and cost-effective biofuel production from microalgae: a review, Int. J. Environ. Sci. Technol. 13 (2016) 2735–2756. doi:10.1007/s13762-016-1020-5.

[242] F. Ahmad Ansari, A. Shriwastav, S. Kumar Gupta, I. Rawat, F. Bux, Exploration of Microalgae Biorefinery by Optimizing Sequential Extraction of Major Metabolites from Scenedesmus obliquus, Ind. Eng. Chem. Res. 56 (2017) 3407–3412. doi:10.1021/acs.iecr.6b04814.

[243] M. van der Spiegel, M.Y. Noordam, H.J. van der Fels-Klerx, Safety of Novel Protein Sources (Insects, Microalgae, Seaweed, Duckweed, and Rapeseed) and Legislative Aspects for Their Application in Food and Feed Production, Compr. Rev. Food Sci. Food Saf. 12 (2013) 662–678. doi:10.1111/1541-4337.12032.

[244] M. Vigani, C. Parisi, E. Rodríguez-Cerezo, M.J. Barbosa, L. Sijtsma, M. Ploeg, C. Enzing, Food and feed products from micro-algae: Market opportunities and challenges for the EU, Trends Food Sci. Technol. 42 (2015) 81–92. doi:10.1016/j.tifs.2014.12.004.

[245] J.S. Ruiz, G. Olivieri, J. De Vree, R. Bosma, P. Willems, J.H. Reith, M.H.M. Eppink, D.M.M. Kleinegris, R.H. Wijffels, M.J. Barbosa, Towards industrial products from microalgae, Energy Environ. Sci. 9 (2016) 3036–3034. doi:10.1039/c6ee01493c.

[246] F. Fasaei, J.H. Bitter, P.M. Slegers, A.J.B. Van Boxtel, Techno-economic evaluation of microalgae harvesting and dewatering systems, Algal Res. 31 (2018) 347–362. doi:10.1016/j.algal.2017.11.038.

[247] L. Xu, P.J. Weathers, X.-R. Xiong, C.-Z. Liu, Microalgal bioreactors: Challenges and opportunities, Eng. Life Sci. 9 (2009) 178–189. doi:10.1002/elsc.200800111.

[248] B. Wang, C.Q. Lan, M. Horsman, Closed photobioreactors for production of microalgal biomasses, Biotechnol. Adv. 30 (2012) 904–912. doi:10.1016/J.BIOTECHADV.2012.01.019.

[249] M. Glemser, M. Heining, J. Schmidt, A. Becker, D. Garbe, R. Buchholz, T. Brück, Application of light-emitting diodes (LEDs) in cultivation of phototrophic microalgae: current state and perspectives, Appl. Microbiol. Biotechnol. 100 (2016) 1077–1088.

doi:10.1007/s00253-015-7144-6.

[250] Y. Sun, Y. Huang, Q. Liao, Q. Fu, X. Zhu, Enhancement of microalgae production by embedding hollow light guides to a flat-plate photobioreactor, Bioresour. Technol. 207 (2016) 31–38. doi:https://doi.org/10.1016/j.biortech.2016.01.136.

[251] N. Söyler, J.L. Goldfarb, S. Ceylan, M.T. Saçan, Renewable fuels from pyrolysis of Dunaliella tertiolecta: An alternative approach to biochemical conversions of microalgae, Energy. 120 (2017) 907–914. doi:10.1016/J.ENERGY.2016.11.146.

[252] B. Gilbert-López, J.A. Mendiola, J. Fontecha, L.A.M. Van Den Broek, L. Sijtsma, A. Cifuentes, M. Herrero, E. Ibáñez, Downstream processing of Isochrysis galbana: a step towards microalgal biorefinery, Green Chem. 17 (2015) 4599–4609. doi:10.1039/c5gc01256b.

[253] J. Li, Y. Liu, J.J. Cheng, M. Mos, M. Daroch, Biological potential of microalgae in China for biorefinery-based production of biofuels and high value compounds, N. Biotechnol. 32 (2015) 588–596. doi:10.1016/J.NBT.2015.02.001.

[254] B.P. Nobre, F. Villalobos, B.E. Barragán, A.C. Oliveira, A.P. Batista, P.A.S.S. Marques, R.L. Mendes, H. Sovová, A.F. Palavra, L. Gouveia, A biorefinery from Nannochloropsis sp. microalga – Extraction of oils and pigments. Production of biohydrogen from the leftover biomass, Bioresour. Technol. 135 (2013) 128–136. doi:10.1016/J.BIORTECH.2012.11.084.

[255] J.A. Posada, L.B. Brentner, A. Ramirez, M.K. Patel, Conceptual design of sustainable integrated microalgae biorefineries: Parametric analysis of energy use, greenhouse gas emissions and techno-economics, Algal Res. 17 (2016) 113–131. doi:10.1016/J.ALGAL.2016.04.022.

[256] A. Naresh Kumar, B. Min, S. Venkata Mohan, Defatted algal biomass as feedstock for short chain carboxylic acids and biohydrogen production in the biorefinery format, Bioresour. Technol. 269 (2018) 408–416.

doi:10.1016/j.biortech.2018.08.059.

[257] R.R. dos Santos, D.M. Moreira, C.N. Kunigami, D.A.G. Aranda, C.M.L.L. Teixeira, Comparison between several methods of total lipid extraction from Chlorella vulgaris biomass, Ultrason. Sonochem. 22 (2015) 95–99. doi:https://doi.org/10.1016/j.ultsonch.2014.05.015.

[258] I.S. Chronakis, M. Madsen, Algal proteins, in: G.O. Phillips, P.A. Williams (Eds.), Handb. Food Proteins, Woodhead Publishing Series in Food Sciences, Technology and Nutrition, 2011: pp. 353–394.

[259] R. Sankaran, P.L. Show, S.Y. Lee, Y.J. Yap, T.C. Ling, Integration process of fermentation and liquid biphasic flotation for lipase separation from Burkholderia cepacia, Bioresour. Technol. 250 (2018) 306–316. doi:https://doi.org/10.1016/j.biortech.2017.11.050.

[260] H.Y. Leong, C.W. Ooi, C.L. Law, A.L. Julkifle, T. Katsuda, P.L. Show, Integration process for betacyanins extraction from peel and flesh of Hylocereus polyrhizus using liquid biphasic electric flotation system and antioxidant activity evaluation, Sep. Purif. Technol. 209 (2019) 193–201. doi:10.1016/j.seppur.2018.07.040.

[261] P.L. Show, C.W. Ooi, M.S. Anuar, A. Ariff, Y.A. Yusof, S.K. Chen, M.S.M. Annuar, T.C. Ling, Recovery of lipase derived from Burkholderia cenocepacia ST8 using sustainable aqueous two-phase flotation composed of recycling hydrophilic organic solvent and inorganic salt, Sep. Purif. Technol. 110 (2013) 112–118. doi:https://doi.org/10.1016/j.seppur.2013.03.018.

[262] N.L.M. Sidek, J.S. Tan, S. Abbasiliasi, F.W.F. Wong, S. Mustafa, A.B. Ariff, Aqueous two-phase flotation for primary recovery of bacteriocin-like inhibitory substance (BLIS) from Pediococcus acidilactici Kp10, J. Chromatogr. B. 1027 (2016) 81–87. doi:https://doi.org/10.1016/j.jchromb.2016.05.024.

[263] W.N. Phong, P.L. Show, W.H. Teh, T.X. Teh, H.M.Y. Lim, N.S. binti Nazri, C.H. Tan,

J.S. Chang, T.C. Ling, Proteins recovery from wet microalgae using liquid biphasic flotation (LBF), Bioresour. Technol. 244 (2017) 1329–1336. doi:10.1016/j.biortech.2017.05.165.

[264] R. Sankaran, S. Manickam, Y.J. Yap, T.C. Ling, J.S. Chang, P.L. Show, Extraction of proteins from microalgae using integrated method of sugaring-out assisted liquid biphasic flotation (LBF) and ultrasound, Ultrason. Sonochem. 48 (2018) 231–239. doi:10.1016/j.ultsonch.2018.06.002.

[265] P.B. Dhamole, P. Mahajan, H. Feng, Sugaring out: A new method for removal of acetonitrile from preparative RP-HPLC eluent for protein purification, Process Biochem. 45 (2010) 1672–1676. doi:https://doi.org/10.1016/j.procbio.2010.06.020.

[266] H. Leinonen, Stress Corrosion Cracking and Life Prediction Evaluation of Austenitic Stainless Steels in Calcium Chloride Solution, CORROSION. 52 (1996) 337–346. doi:10.5006/1.3292121.

[267] I.M. Valente, L.M. Gonçalves, J.A. Rodrigues, Another glimpse over the salting-out assisted liquid-liquid extraction in acetonitrile/water mixtures, J. Chromatogr. A. 1308 (2013) 58–62. doi:10.1016/j.chroma.2013.08.014.

[268] E. Günerken, E. D'Hondt, M.H.M. Eppink, L. Garcia-Gonzalez, K. Elst, R.H. Wijffels, Cell disruption for microalgae biorefineries, Biotechnol. Adv. 33 (2015) 243–260. doi:https://doi.org/10.1016/j.biotechadv.2015.01.008.

[269] M.D.A. Zbinden, B.S.M. Sturm, R.D. Nord, W.J. Carey, D. Moore, H. Shinogle, S.M. Stagg-Williams, Pulsed electric field (PEF) as an intensification pretreatment for greener solvent lipid extraction from microalgae, Biotechnol. Bioeng. 110 (2013) 1605–1615. doi:10.1002/bit.24829.

[270] M. Mubarak, A. Shaija, T. V Suchithra, Ultrasonication: An effective pre-treatment method for extracting lipid from Salvinia molesta for biodiesel production, Resour.

Technol. 2 (2016) 126–132. doi:https://doi.org/10.1016/j.reffit.2016.07.005.

[271] E. Roselló-Soto, F.J. Barba, O. Parniakov, C.M. Galanakis, N. Lebovka, N. Grimi, E. Vorobiev, High Voltage Electrical Discharges, Pulsed Electric Field, and Ultrasound Assisted Extraction of Protein and Phenolic Compounds from Olive Kernel, Food Bioprocess Technol. 8 (2015) 885–894. doi:10.1007/s11947-014-1456-x.

[272] E.W. Becker, Micro-algae as a source of protein, Biotechnol. Adv. 25 (2007) 207–210. doi:https://doi.org/10.1016/j.biotechadv.2006.11.002.

[273] S.O. Lourenço, E. Barbarino, P.L. Lavín, U.M.L. Marquez, E. Aidar, Distribution of intracellular nitrogen in marine microalgae: Calculation of new nitrogen-to-protein conversion factors, Eur. J. Phycol. 39 (2004) 17–32. doi:10.1080/0967026032000157156.

[274] C. Safi, M. Charton, O. Pignolet, F. Silvestre, C. Vaca-Garcia, P.-Y. Pontalier, Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors, J. Appl. Phycol. 25 (2013) 523–529. doi:10.1007/s10811-012-9886-1.

[275] P.L. Chew, M.S.M. Annuar, P.L. Show, T.C. Ling, Extractive bioconversion of poly-ε-caprolactone by Burkholderia cepacia lipase in an aqueous two-phase system, Biochem. Eng. J. 101 (2015) 9–17. doi:10.1016/j.bej.2015.04.015.

[276] P. Selvakumar, T.C. Ling, S. Walker, A. Lyddiatt, A practical implementation and exploitation of ATPS for intensive processing of biological feedstock: A novel approach for heavily biological feedstock loaded ATPS, Sep. Purif. Technol. 75 (2010) 323–331. doi:https://doi.org/10.1016/j.seppur.2010.08.022.

[277] P.L. Show, C.P. Tan, M.S. Anuar, A. Ariff, Y.A. Yusof, S.K. Chen, T.C. Ling, Direct recovery of lipase derived from Burkholderia cepacia in recycling aqueous two-phase flotation, Sep. Purif. Technol. 80 (2011) 577–584. doi:10.1016/J.SEPPUR.2011.06.013.

[278] J. Azmir, I.S.M. Zaidul, M.M. Rahman, K.M. Sharif, A. Mohamed, F. Sahena, M.H.A. Jahurul, K. Ghafoor, N.A.N. Norulaini, A.K.M. Omar, Techniques for extraction of bioactive compounds from plant materials: A review, J. Food Eng. 117 (2013) 426–436. doi:https://doi.org/10.1016/j.jfoodeng.2013.01.014.

[279] F. Chemat, N. Rombaut, A. Meullemiestre, M. Turk, S. Perino, A.-S. Fabiano-Tixier, M. Abert-Vian, Review of Green Food Processing techniques. Preservation, transformation, and extraction, Innov. Food Sci. Emerg. Technol. 41 (2017) 357–377. doi:https://doi.org/10.1016/j.ifset.2017.04.016.

[280] J. Szydłowski, M. Szykuła, Isotope effect on miscibility of acetonitrile and water, Fluid Phase Equilib. 154 (1999) 79–87. doi:https://doi.org/10.1016/S0378-3812(98)00438-5.

[281] B. Wang, T. Ezejias, H. Feng, H. Blaschek, Sugaring-out: A novel phase separation and extraction system, Chem. Eng. Sci. 63 (2008) 2595–2600. doi:https://doi.org/10.1016/j.ces.2008.02.004.

[282] P.B. Dhamole, S. Chavan, R.G. Patil, H. Feng, M. Bule, P. Kinninge, Extraction of p-coumaric acid from agricultural residues and separation using `sugaring out', Korean J. Chem. Eng. 33 (2016) 1860–1864. doi:10.1007/s11814-016-0020-y.

[283] G. de Brito Cardoso, T. Mourão, F.M. Pereira, M.G. Freire, A.T. Fricks, C.M.F. Soares, Á.S. Lima, Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin, Sep. Purif. Technol. 104 (2013) 106–113. doi:https://doi.org/10.1016/j.seppur.2012.11.001.

[284] S. V Pakhale, M.D. Vetal, V.K. Rathod, Separation of Bromelain by Aqueous Two Phase Flotation, Sep. Sci. Technol. 48 (2013) 984–989. doi:10.1080/01496395.2012.712596.

[285] P. Bi, H. Dong, J. Dong, The recent progress of solvent sublation, J. Chromatogr. A. 1217 (2010) 2716–2725. doi:https://doi.org/10.1016/j.chroma.2009.11.020.

[286] O. Edenhofer, R. Pichs-Madruga, Y. Sokona, E. Farahani, S. Kadner, K. Seyboth, Summary for policymakers, in: Clim. Chang. 2014 Mitig. Clim. Chang. Contrib. Work. Gr. III to Fifth Assess. Rep. Intergov. Panel Clim. Chang., Cambridge University Press, Cambridge, 2014: pp. 1–32.

[287] J.-M. Robine, S.L.K. Cheung, S. Le Roy, H. Van Oyen, C. Griffiths, J.-P. Michel, F.R. Herrmann, Death toll exceeded 70,000 in Europe during the summer of 2003, C. R. Biol. 331 (2008) 171–178. doi:https://doi.org/10.1016/j.crvi.2007.12.001.

[288] A.S. Nizami, M. Rehan, M. Waqas, M. Naqvi, O.K.. Ouda, K. Shahzad, R. Miandad, M.Z. Khan, M. Syamsiro, I.M.I. Ismail, D. Pant, Waste biorefineries: Enabling circular economies in developing countries, Bioresour. Technol. 241 (2017) 1101–1117. doi:10.1016/J.BIORTECH.2017.05.097.

[289] S. Venkata Mohan, G.N. Nikhil, P. Chiranjeevi, C. Nagendranatha Reddy, M.V. Rohit, A.N. Kumar, O. Sarkar, Waste biorefinery models towards sustainable circular bioeconomy: Critical review and future perspectives, Bioresour. Technol. 215 (2016) 2–12. doi:10.1016/J.BIORTECH.2016.03.130.

[290] K.H. Shariat Panahi, H. Kazemi Shariat Panahi, M. Dehhaghi, J.E. Kinder, T. Chukwuemeka Ezeji, A review on green liquid fuels for the transportation sector: a prospect of microbial solutions to climate change, Biofuel Res. J. 23 (2019) 995–1024. doi:10.18331/BRJ2019.6.3.2.

[291] R. Chandra, H.M.N. Iqbal, G. Vishal, H.-S. Lee, S. Nagra, Algal biorefinery: A sustainable approach to valorize algal-based biomass towards multiple product recovery, Bioresour. Technol. 278 (2019) 346–359. doi:10.1016/J.BIORTECH.2019.01.104.

[292] A.T. Ubando, C.B. Felix, W.-H. Chen, Biorefineries in circular bioeconomy: A comprehensive review, Bioresour. Technol. 299 (2020) 122585. doi:10.1016/J.BIORTECH.2019.122585.

[293] Á. Cancela, X. Álvarez, Á. Sánchez, L. Ortiz, L. Somoza, Microalgae cultivation and harvesting for bioenergy production, Bioresour. Technol. Reports. 8 (2019) 100333. doi:10.1016/J.BITEB.2019.100333.

[294] A.A. Arnold, B. Genard, F. Zito, R. Tremblay, D.E. Warschawski, I. Marcotte, Identification of lipid and saccharide constituents of whole microalgal cells by 13C solid-state NMR, Biochim. Biophys. Acta - Biomembr. 1848 (2015) 369–377. doi:10.1016/J.BBAMEM.2014.07.017.

[295] W. Wu, J.-S. Chang, Integrated algal biorefineries from process systems engineering aspects: A review, Bioresour. Technol. 291 (2019) 121939. doi:10.1016/J.BIORTECH.2019.121939.

[296] C.G. Khoo, Y.K. Dasan, M.K. Lam, K.T. Lee, Algae biorefinery: Review on a broad spectrum of downstream processes and products, Bioresour. Technol. 292 (2019) 121964. doi:10.1016/J.BIORTECH.2019.121964.

[297] K.S. Khoo, H.Y. Leong, K.W. Chew, J. Lim, T.C. Ling, P.L. Show, H. Yen, Liquid Biphasic System : A Recent Bioseparation Technology, Processes. 8 (2020) 1–22. doi:https://doi.org/10.3390/pr8020149.

[298] S.R. Chia, K.W. Chew, P.L. Show, M. Sivakumar, T.C. Ling, Y. Tao, Isolation of protein from Chlorella sorokiniana CY1 using liquid biphasic flotation assisted with sonication through sugaring-out effect, J. Oceanol. Limnol. 37 (2019) 898–908. doi:10.1007/s00343-019-8246-2.

[299] K.W. Chew, T.C. Ling, P.L. Show, Recent Developments and Applications of Three-Phase Partitioning for the Recovery of Proteins, Sep. Purif. Rev. 48 (2019) 52–64. doi:10.1080/15422119.2018.1427596.

[300] A.M. Lizzul, A. Lekuona-Amundarain, S. Purton, L.C. Campos, Characterization of chlorella sorokiniana, UTEX 1230, Biology (Basel). 7 (2018) 1–12. doi:10.3390/biology7020025.

[301] Y. Chen, S. Vaidyanathan, A simple, reproducible and sensitive spectrophotometric method to estimate microalgal lipids, Anal. Chim. Acta. 724 (2012) 67–72. doi:10.1016/J.ACA.2012.02.049.

[302] A. Reddy, A.B. Majumder, Use of a Combined Technology of Ultrasonication , Three-Phase Partitioning , and Aqueous Enzymatic Oil ... Use of a Combined Technology of Ultrasonication , Three-Phase Partitioning , and Aqueous Enzymatic Oil Extraction for the Extraction of Oil from Spi, 2014 (2016). doi:10.1155/2014/740631.

[303] K. Mulchandani, J.R. Kar, R.S. Singhal, Extraction of Lipids from Chlorella saccharophila Using High-Pressure Homogenization Followed by Three Phase Partitioning, Appl. Biochem. Biotechnol. 176 (2015) 1613–1626. doi:10.1007/s12010-015-1665-4.

[304] J.K. Yan, Y.Y. Wang, W.Y. Qiu, H. Ma, Z. Bin Wang, J.Y. Wu, Three-phase partitioning as an elegant and versatile platform applied to nonchromatographic bioseparation processes, Crit. Rev. Food Sci. Nutr. 58 (2018) 2416–2431. doi:10.1080/10408398.2017.1327418.

[305] D.C. Panadare, V.K. Rathod, Three phase partitioning for extraction of oil: A review, Trends Food Sci. Technol. 68 (2017) 145–151. doi:10.1016/J.TIFS.2017.08.004.

[306] C. Qiu, Y. He, Z. Huang, S. Li, J. Huang, M. Wang, B. Chen, Lipid extraction from wet Nannochloropsis biomass via enzyme-assisted three phase partitioning, Bioresour. Technol. 284 (2019) 381–390. doi:10.1016/J.BIORTECH.2019.03.148.

[307] P.D. Patil, G.D. Yadav, Application of microwave assisted three phase partitioning method for purification of laccase from Trametes hirsuta, Process Biochem. 65 (2018) 220–227. doi:10.1016/J.PROCBIO.2017.10.006.

[308] R. Ravanfar, A.M. Tamadon, M. Niakousari, Optimization of ultrasound assisted extraction of anthocyanins from red cabbage using Taguchi design method, J. Food

Sci. Technol. 52 (2015) 8140–8147. doi:10.1007/s13197-015-1880-6.

[309] C. Safi, A.V. Ursu, C. Laroche, B. Zebib, O. Merah, P.-Y. Pontalier, C. Vaca-Garcia, Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods, Algal Res. 3 (2014) 61–65. doi:10.1016/J.ALGAL.2013.12.004.

[310] J.A. Gerde, M. Montalbo-Lomboy, L. Yao, D. Grewell, T. Wang, Evaluation of microalgae cell disruption by ultrasonic treatment, Bioresour. Technol. 125 (2012) 175–181. doi:10.1016/J.BIORTECH.2012.08.110.

[311] S.R. Chia, K.Y. Mak, Y.J. Khaw, N. Suhaidi, K.W. Chew, P.L. Show, An efficient and rapid method to extract and purify protein – Liquid Triphasic Flotation system, Bioresour. Technol. 294 (2019) 122158. doi:10.1016/J.BIORTECH.2019.122158.

[312] P.L. Show, C.P. Tan, M.S. Anuar, A. Ariff, Y.A. Yusof, S.K. Chen, T.C. Ling, Primary recovery of lipase derived from Burkholderia cenocepacia strain ST8 and recycling of phase components in an aqueous two-phase system, Biochem. Eng. J. 60 (2012) 74–80. doi:10.1016/J.BEJ.2011.10.005.

[313] R.V. Sreeharsha, S. Venkata Mohan, Symbiotic integration of bioprocesses to design a self-sustainable life supporting ecosystem in a circular economy framework, Bioresour. Technol. 326 (2021) 124712. doi:https://doi.org/10.1016/j.biortech.2021.124712.

[314] J. Rajesh Banu, Preethi, S. Kavitha, M. Gunasekaran, G. Kumar, Microalgae based biorefinery promoting circular bioeconomy-techno economic and life-cycle analysis, Bioresour. Technol. 302 (2020) 122822. doi:https://doi.org/10.1016/j.biortech.2020.122822.

[315] J. Sherwood, The significance of biomass in a circular economy, Bioresour. Technol. 300 (2020) 122755. doi:https://doi.org/10.1016/j.biortech.2020.122755.

[316] S.P. Singh, P. Singh, Effect of temperature and light on the growth of algae species: A review, Renew. Sustain. Energy Rev. 50 (2015) 431–444.

doi:https://doi.org/10.1016/j.rser.2015.05.024.

[317] G. López, C. Yate, F.A. Ramos, M.P. Cala, S. Restrepo, S. Baena, Production of Polyunsaturated Fatty Acids and Lipids from Autotrophic, Mixotrophic and Heterotrophic cultivation of Galdieria sp. strain USBA-GBX-832, Sci. Rep. 9 (2019) 1–13. doi:10.1038/s41598-019-46645-3.

[318] S. Kawasaki, K. Yamazaki, T. Nishikata, T. Ishige, H. Toyoshima, A. Miyata, Photooxidative stress-inducible orange and pink water-soluble astaxanthin-binding proteins in eukaryotic microalga, Commun. Biol. 3 (2020) 1–9. doi:10.1038/s42003-020-01206-7.

[319] S. Li, T. Hu, Y. Xu, J. Wang, R. Chu, Z. Yin, F. Mo, L. Zhu, A review on flocculation as an efficient method to harvest energy microalgae: Mechanisms, performances, influencing factors and perspectives, Renew. Sustain. Energy Rev. 131 (2020) 110005. doi:https://doi.org/10.1016/j.rser.2020.110005.

[320] W.A. Stirk, P. Bálint, M. Vambe, C. Lovász, Z. Molnár, J. van Staden, V. Ördög, Effect of cell disruption methods on the extraction of bioactive metabolites from microalgal biomass, J. Biotechnol. 307 (2020) 35–43. doi:https://doi.org/10.1016/j.jbiotec.2019.10.012.

[321] J. Trivedi, M. Aila, D.P. Bangwal, S. Kaul, M.O. Garg, Algae based biorefinery—How to make sense?, Renew. Sustain. Energy Rev. 47 (2015) 295–307. doi:https://doi.org/10.1016/j.rser.2015.03.052.

[322] G.Y. Yew, K.W. Chew, M.A. Malek, Y.C. Ho, W.H. Chen, T.C. Ling, P.L. Show, Hybrid liquid biphasic system for cell disruption and simultaneous lipid extraction from microalgae Chlorella sorokiniana CY-1 for biofuel production, Biotechnol. Biofuels. 12 (2019) 252. doi:10.1186/s13068-019-1591-8.

[323] X.-F. Zhang, X. Wang, G.-H. Luo, Ultrasound-assisted Three Phase Partitioning of phycocyanin from Spirulina platensis, Eur. J. Pure Appl. Chem. 4 (2017) 1–15.

[324] S. Varakumar, K.V. Umesh, R.S. Singhal, Enhanced extraction of oleoresin from ginger (Zingiber officinale) rhizome powder using enzyme-assisted three phase partitioning, Food Chem. 216 (2017) 27–36. doi:https://doi.org/10.1016/j.foodchem.2016.07.180.

[325] M. Gagaoua, N. Boucherba, A. Bouanane-Darenfed, F. Ziane, S. Nait-Rabah, K. Hafid, H.-R. Boudechicha, Three-phase partitioning as an efficient method for the purification and recovery of ficin from Mediterranean fig (Ficus carica L.) latex, Sep. Purif. Technol. 132 (2014) 461–467. doi:https://doi.org/10.1016/j.seppur.2014.05.050.

[326] A.G. Waghmare, M.K. Salve, J.G. LeBlanc, S.S. Arya, Concentration and characterization of microalgae proteins from Chlorella pyrenoidosa, Bioresour. Bioprocess. 3 (2016) 16. doi:10.1186/s40643-016-0094-8.

[327] K.W. Chew, S.R. Chia, S.Y. Lee, L. Zhu, P.L. Show, Enhanced microalgal protein extraction and purification using sustainable microwave-assisted multiphase partitioning technique, Chem. Eng. J. 367 (2019) 1–8. doi:10.1016/J.CEJ.2019.02.131.

[328] A.K. Koyande, K.W. Chew, J.W. Lim, M.K. Lam, Y.C. Ho, P.L. Show, Biorefinery of Chlorella sorokiniana using ultra sonication assisted liquid triphasic flotation system, Bioresour. Technol. 303 (2020) 122931. doi:10.1016/j.biortech.2020.122931.

[329] M. Patel, S. Das, J. Thakur, GC-MS-Based Analysis of Methanol: Chloroform-extracted Fatty Acids from Plant Tissues, Bio-Protocol. 8 (2018) 1–9. doi:10.21769/bioprotoc.3014.

[330] R. Sankaran, P.L. Show, Y.J. Yap, H.L. Lam, T.C. Ling, G.-T. Pan, T.C.-K. Yang, Sustainable approach in recycling of phase components of large scale aqueous two-phase flotation for lipase recovery, J. Clean. Prod. 184 (2018) 938–948. doi:10.1016/J.JCLEPRO.2018.02.301.

[331] V.M. Jain, G.N. Karibasappa, A.S. Dodamani, G.V. Mali, Estimating the carbohydrate content of various forms of tobacco by phenol - sulfuric acid method, J. Educ. Health Promot. 6 (2017) 90. doi:10.4103/jehp.jehp.

[332] R. Gaur, A. Sharma, S.K. Khare, M.N. Gupta, A novel process for extraction of edible oils: Enzyme assisted three phase partitioning (EATPP), Bioresour. Technol. 98 (2007) 696–699. doi:https://doi.org/10.1016/j.biortech.2006.01.023.

[333] S. Shah, A. Sharma, M.N. Gupta, Extraction of oil from Jatropha curcas L. seed kernels by enzyme assisted three phase partitioning, Ind. Crops Prod. 20 (2004) 275–279. doi:https://doi.org/10.1016/j.indcrop.2003.10.010.

[334] K. Mondal, A. Sharma, M.N. Gupta, Three phase partitioning of starch and its structural consequences, Carbohydr. Polym. 56 (2004) 355–359. doi:https://doi.org/10.1016/j.carbpol.2004.03.004.

[335] K. Mulchandani, J.R. Kar, R.S. Singhal, Extraction of Lipids from Chlorella saccharophila Using High-Pressure Homogenization Followed by Three Phase Partitioning, Appl. Biochem. Biotechnol. 176 (2015) 1613–1626. doi:10.1007/s12010-015-1665-4.

[336] A. Sharma, M.N. Gupta, Three phase partitioning of carbohydrate polymers: separation and purification of alginates, Carbohydr. Polym. 48 (2002) 391–395. doi:https://doi.org/10.1016/S0144-8617(01)00313-7.

[337] H.Y. Leong, C.W. Ooi, C.L. Law, A.L. Julkifle, T.C. Ling, P.L. Show, Application of liquid biphasic flotation for betacyanins extraction from peel and flesh of Hylocereus polyrhizus and antioxidant activity evaluation, Sep. Purif. Technol. 201 (2018) 156–166.

[338] S.H. Hadiyanto, Response surface optimization of ultrasound assisted extraction (UAE) of phycocyanin from microalgae Spirulina platensis, Emirates J. Food Agric. (2016). doi:10.9755/ejfa.2015-05-193.

[339] K.S. Khoo, K.W. Chew, G.Y. Yew, S. Manickam, C.W. Ooi, P.L. Show, Integrated ultrasound-assisted liquid biphasic flotation for efficient extraction of astaxanthin from Haematococcus pluvialis, Ultrason. Sonochem. 67 (2020). doi:https://doi.org/10.1016/j.ultsonch.2020.105052.

[340] A. Sharma, K. Mondal, M.N. Gupta, Some studies on characterization of three phase partitioned chitosan, Carbohydr. Polym. 52 (2003) 433–438. doi:https://doi.org/10.1016/S0144-8617(03)00002-X.

[341] S. V Pakhale, S.S. Bhagwat, Purification of serratiopeptidase from Serratia marcescens NRRL B 23112 using ultrasound assisted three phase partitioning, Ultrason. Sonochem. 31 (2016) 532–538. doi:https://doi.org/10.1016/j.ultsonch.2016.01.037.

[342] G.Y. Yew, K.S. Khoo, W.Y. Chia, Y.-C. Ho, C.L. Law, H.Y. Leong, P.L. Show, A novel lipids recovery strategy for biofuels generation on microalgae Chlorella cultivation with waste molasses, J. Water Process Eng. 38 (2020) 101665. doi:https://doi.org/10.1016/j.jwpe.2020.101665.

[343] E. Al-Gharibeh, R. Leathers, K. Kumar, C.-J. Sung, Ignition and combustion characteristics of decanoic acid derived alkyl esters in a fuel ignition tester, Fuel. 276 (2020) 117982. doi:https://doi.org/10.1016/j.fuel.2020.117982.

[344] D. Harper, A. Young, C.-E. McNaught, The physiology of wound healing, Surg. 32 (2014) 445–450. doi:https://doi.org/10.1016/j.mpsur.2014.06.010.

[345] C. Dunnill, T. Patton, J. Brennan, J. Barrett, M. Dryden, J. Cooke, D. Leaper, N.T. Georgopoulos, Reactive oxygen species (ROS) and wound healing: the functional role of ROS and emerging ROS-modulating technologies for augmentation of the healing process, Int. Wound J. 14 (2017) 89–96. doi:https://doi.org/10.1111/iwj.12557.

[346] G. Fiakos, Z. Kuang, E. Lo, Improved skin regeneration with acellular fish skin

grafts, Eng. Regen. 1 (2020) 95–101. doi:https://doi.org/10.1016/j.engreg.2020.09.002.

[347] K. Shanthi Kumari, P. Shivakrishna, V.S.R. Ganduri, Wound healing Activities of the bioactive compounds from Micrococcus sp. OUS9 isolated from marine water, Saudi J. Biol. Sci. 27 (2020) 2398–2402. doi:https://doi.org/10.1016/j.sjbs.2020.05.007.

[348] A. Luiza Astolfi, A. Rempel, V.A.F. Cavanhi, M. Alves, K.M. Deamici, L.M. Colla, J.A.V. Costa, Simultaneous saccharification and fermentation of Spirulina sp. and corn starch for the production of bioethanol and obtaining biopeptides with high antioxidant activity, Bioresour. Technol. 301 (2020) 122698. doi:https://doi.org/10.1016/j.biortech.2019.122698.

[349] N.P.A. Syarina, G. Karthivashan, F. Abas, P. Arulselvan, S. Fakurazi, Wound healing potential of Spirulina platensis extracts on Human Dermal Fibroblast cells, EXCLI J. 14 (2015) 385–393. doi:dx.doi.org/10.17179/excli2014-697.

[350] G. Yang, Z. Xiao, H. Long, K. Ma, J. Zhang, X. Ren, J. Zhang, Assessment of the characteristics and biocompatibility of gelatin sponge scaffolds prepared by various crosslinking methods, Sci. Rep. 8 (2018) 1616. doi:10.1038/s41598-018-20006-y.

[351] S.-M. Jung, S.K. Min, H.C. Lee, Y.S. Kwon, M.H. Jung, H.S. Shin, Spirulina-PCL Nanofiber Wound Dressing to Improve Cutaneous Wound Healing by Enhancing Antioxidative Mechanism, J. Nanomater. 2016 (2016) 6135727. doi:10.1155/2016/6135727.

[352] L. Zhang, L. Chen, Y. Xiang, Z. Liu, X. Mao, L. Zhang, L. Deng, Y. Zhang, L. Cheng, X. Sun, W. Cui, Multifunctional integrally-medicalized hydrogel system with internal synergy for efficient tissue regeneration, Chem. Eng. J. 406 (2021) 126839. doi:https://doi.org/10.1016/j.cej.2020.126839.

[353] M. Awang, M. Firdaus, M. Busra, S. Chowdhury, N. Fadilah, W. Wan Hamirul, M. Reusmaazran, M. Aminuddin, B. Ruszymah, Cytotoxic evaluation of biomechanically improved crosslinked ovine collagen on human dermal fibroblasts, Biomed. Mater. Eng. 24 (2014) 1715–1724. doi:10.3233/BME-140983.

[354] A. Bigi, G. Cojazzi, S. Panzavolta, K. Rubini, N. Roveri, Mechanical and thermal properties of gelatin films at different degrees of glutaraldehyde crosslinking, Biomaterials. 22 (2001) 763–768. doi:10.1016/S0142-9612(00)00236-2.

[355] C. Chen, F. Liu, Z. Yu, Y. Ma, H.D. Goff, F. Zhong, Improvement in physicochemical properties of collagen casings by glutaraldehyde cross-linking and drying temperature regulating, Food Chem. 318 (2020) 126404. doi:10.1016/j.foodchem.2020.126404.

[356] J. Lin, D. Pan, Y. Sun, C. Ou, Y. Wang, J. Cao, The modification of gelatin films: Based on various cross-linking mechanism of glutaraldehyde at acidic and alkaline conditions, Food Sci. Nutr. 7 (2019) 4140–4146. doi:10.1002/fsn3.1282.

[357] A. Beishenaliev, S.S. Lim, K.Y. Tshai, P.S. Khiew, H.N. Moh'd Sghayyar, H.S. Loh, Fabrication and preliminary in vitro evaluation of ultraviolet-crosslinked electrospun fish scale gelatin nanofibrous scaffolds, J. Mater. Sci. Mater. Med. 30 (2019). doi:10.1007/s10856-019-6264-4.

[358] H.N.M. Sghayyar, S.S. Lim, I. Ahmed, J.Y. Lai, X.Y. Cheong, Z.W. Chong, A.F.X. Lim, H.S. Loh, Fish biowaste gelatin coated phosphate-glass fibres for wound-healing application, Eur. Polym. J. 122 (2020). doi:10.1016/j.eurpolymj.2019.109386.

[359] E. Bari, C.R. Arciola, B. Vigani, B. Crivelli, P. Moro, G. Marrubini, M. Sorrenti, L. Catenacci, G. Bruni, T. Chlapanidas, E. Lucarelli, S. Perteghella, M.L. Torre, In Vitro Effectiveness of Microspheres Based on Silk Sericin and Chlorella vulgaris or Arthrospira platensis for Wound Healing Applications, Materials (Basel). 10 (2017).

doi:10.3390/ma10090983.

[360] E. Machmud, M. Ruslin, R. Waris, R.A. Asse, A.M. Qadafi, H. Achmad, Effect of the application of Chlorella vulgaris ointment to the number of fibroblast cells as an indicator of wound healing in the soft tissue of pig ears, Pesqui. Bras. Odontopediatria Clin. Integr. 20 (2020). doi:10.1590/pboci.2020.032.

[361] N. Zailan, A.H. Abdul Rashid, S. Das, N.A. Abdul Mokti, J. Hassan Basri, S.L. Teoh, W.Z. Wan Ngah, Y.A. Mohd Yusof, Comparison of Chlorella vulgaris dressing and sodium alginate dressing: An experimental study in rats, Clin. Ter. 161 (2010) 515–521.

[362] S. Veeraperumal, H.-M. Qiu, S.-S. Zeng, W.-Z. Yao, B.-P. Wang, Y. Liu, K.-L. Cheong, Polysaccharides from Gracilaria lemaneiformis promote the HaCaT keratinocytes wound healing by polarised and directional cell migration, Carbohydr. Polym. 241 (2020) 116310. doi:https://doi.org/10.1016/j.carbpol.2020.116310.

[363] P. Liu, J.-W. Choi, M.-K. Lee, Y.-H. Choi, T.-J. Nam, Wound Healing Potential of Spirulina Protein on CCD-986sk Cells, Mar. Drugs. 17 (2019). doi:10.3390/md17020130.